GENETIC PROGNOSTIC FACTORS IN ACUTE MYELOID LEUKEMIA

PhD dissertation

Magdalena Renata Koszarska

Eötvös Loránd University, Faculty of Science

Doctoral School of Biology

Classical and Molecular Genetics PhD Program

Leader of the PhD School: Anna Erdei DSc, member of HAS

Leader of PhD Program: Tibor Vellai DSc

Supervisors: Hajnalka Andrikovics M.D., PhD

Váradi András DSc

Eötvös Loránd University, Faculty of Science

Department of Genetics

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ABBREVIATIONS

2-HG	2-hydroxy glutarate
5D3	anti-BCRP antibody, clone 5D3
ABC	ATP-binding cassette
ABCG2	ATP-binding cassette, sub-family G, member 2
ABL1	Abelson tyrosine kinase 1
AF	allele frequency
alpha-KG	alpha ketoglutarate
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
AS-PCR	allele specific polymerase chain reaction
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
BM	bone marrow
bp	base pair
BXP-21	anti-ABCG2 antibody, clone BXP-21
BXP-34	anti-ABCG2 antibody, clone BXP-34
CBFβ	core binding factor beta subunit
CBFβ-MYH11	core binding factor beta subunit-myosin, heavy chain 11 fusion gene
CD34	cluster of differentiation 34
cDNA	complementary deoxyribonucleic acid
CEBPA	CCAAT enhancer binding protein alpha
CML	chronic myeloid leukemia
CI	confidence interval
Cp	crossing point
CR	complete remission
DFS	disease free survival
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FAB	French-American-British classification

FISH	fluorescense in situ hybridyzation
FLT3	*fms*-like tyrosine kinase 3
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
HLA	human leukocyte antigen
HR	hazard ratio
HRM	high resolution melting
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
IDH1/2	isocitrate dehydrogenase 1 or 2
IgG	immunoglobulin G
ITD	internal tandem duplication
JMD	juxta-membrane domain
MDS	myelodysplastic syndrome
MDR1	multidrug resistance protein 1
MPN	myeloproliferative neoplasm
mRNA	messenger ribonucleic acid
MRP1	multidrug resistance protein 1
NK-AML	normal karyotype acute myeloid leukemia
NOS	not otherwise specified
NPM1	nucleophosmin 1
OR	odds ratio
OS	overall survival
PB	peripheral blood
PCR	polymerase chain reaction
PLT	platelet
PML-RARA	promyelocytic leukemia-retinoic acid receptor alpha fusion gene
RBC	red blood cell
RNA	ribonucleic acid
RUNX1-RUNX1T1	runt-related transcription factor1- runt-related transcription factor1; translocated to, 1(cyclin D-related) fusion gene
Q-PCR	quantitative polymerase chain reaction
SLS	sample loading solution
SNP	single nucleotide polimorphism

SPSS	Statistical Package for the Social Science version 13.0
SSC	side scatter
t-AML	therapy-related myeloid neoplasm
TET1/2	tet methylcytosine dioxygenase 1 or 2
TKD	tyrosine kinase domain
TKI	tyrosine kinase inhibitor
UPD	uniparental disomy
WBC	white blood cell
WT	wild type

1. INTRODUCTION

1.1 Clinical characteristics of AML

The term, leukemia was described by Rudolf Virchow for the first time in 1856 (Piller; 2001). The name, leukemia originates from the ancient Greek words: *leukós haima*, which means ″white blood″. Leukemia is a malignant disorder characterized by increased proliferation and blocked differentiation of a hematopoietic stem cell clone (Hoffbrand; 2001). Leukemia may be divided into two subgroups: lymphoid and myeloid leukemias according to affected cell lineage and further into chronic and acute leukemias, depending on ratio of immature blast cells in the bone marrow. Acute leukemias manifest suddenly with severe signs and symptoms, and progress rapidly without treatment, while chronic disorders usually start smoldering (e.g. only laboratory changes, without any severe symptoms) with no or slow progression during the first years after diagnosis.

Acute myeloid leukemia (AML) is a disorder of myeloid hematopoietic stem cells (HSC) with the presence of more than 20% of myeloid blast cells in the bone marrow. It occurs in 1/10 000 persons/year, in all age groups, but it is more common in the elderly. It is observed equally in both genders (Hoffbrand; 2001). An increased incidence of AML has been noted among individuals with genetic disorders such as: Down´s syndrome, Fanconi´s anemia, Bloom syndrome or ataxia-telangiectasia (Litzow; 2001). Besides the above mentioned factors, there are also environmental factors that are thought to cause DNA damage and hasten the onset of AML such as: smoking (the only proven lifestyle-related risk factor in AML), certain chemical exposure (long-term exposure to benzene), ionizing radiation (nuclear bomb exposure and nuclear reactor accidents such as Chernobyl), cancer treatment (patients receiving certain chemotherapy, including alkylating agents and topoisemarase II inhibitors), certain other myeloid clonal disorders (myelodysplastic syndrome or myleproliferative neoplasm) and family history (Litzow; 2001). Although RNA tumor viruses are known to cause AML in some animal models (cat), their role in human AML has not been proven yet.

AML may arise *de novo* or as after the effects (secondary) of other hematological disease (myelodysplasia or myeloproliferative disorders) or previous chemotherapy (Hoffbrand; 2001). Both types are characterized by highly heterogeneous genetic background, including more than 200 recurrent cytogenetic abnormalities and more than 23 molecular genetic aberrations (Dohner; 2010, Ley; 2013) AML is classified into

particular groups, depending on morphology, immunophenotype describing the level of differentiation of the immature myeloid blast cells, genetics and prognostic outcome. One of the first grouping was the French-American-British (FAB) classification (see Table 1) (Bennett; 1976).

Table 1. The French-American-British (FAB) classification of AML.

FAB subtype	NAME
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4eo	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

According to the FAB classification, AML was divided into 8 subgroups, M0 through M7, according to morphology, cellularity, blast percentage and cytochemistry (Cheson; 2003). Precursors of white blood cells (WBC) are responsible for M0 through M5 subtypes, precursors of red blood cells (RBC) for M6 and precursors of platelets (PLT) for M7. Besides the precursors, patients are also grouped according to other symptoms, for example; bleeding and blood clotting problems are specific for M3 AML subtype, also known as acute promyelocytic leukemia (APL). However, FAB classification did not include genetics, cytogenetics, or clinical outcome.

Cytogenetics and molecular genetics appeared to be important prognostic factors in several leukemias. The recent World Health Organization (WHO 2008) classification was based on new insights with regards to cytogenetics and molecular genetics beside morphology and immunologic markers, (Ley; 2008, Vardiman; 2009) (Table 2). The first category of the WHO 2008 classification is AML with recurrent genetic abnormalities. The reciprocal translocations or inversions, such as: t(8;21), inv(16) or t(16;16) and t(15;17) are the most frequent cytogenetic abnormalities in AML. They result in the formation of fusion genes, which are responsible for the initiation of leukemogenesis. These recurrent cytogenetic abnormalities are characterized by favorable treatment outcome. The

translocation [t(8;21)(q22;q22)], resulting in the formation of *RUNX1-RUNX1T1* (runt-related transcription factor1- runt-related transcription factor 1; translocated to, 1 (cyclin D-related) fusion gene) also known as *AML1-ETO* fusion gene, is one of the most frequent cytogenetic aberrations in AML with frequency of 5-12%.

It has the highest frequency in FAB classification M2. The inversion of chromosome 16 [inv(16)(p13.1q22)] or the reciprocal translocation between chromosomes 16 [t(16;16)(p13;q22)], resulting in the formation of *CBFβ-MYH11* (core binding factor beta subunit-myosin, heavy chain 11 fusion) fusion gene, occurs in approximately 10% of all AML cases, predominantly in younger patients and FAB M4eo morphology. The third most frequent translocation, occurring mostly in adult AML patients, is t(15;17)(q22;q12) also called *PML-RARA* (promyelocytic leukemia-retinoic acid receptor alpha fusion gene) with a frequency of 5-8%. It is found in FAB classification M3 (APL). PML-RARA fusion transcript positive APL is specifically sensitive to treatment with all-trans retinoic acids (ATRA), which acts as a differentiating agent. Similarly to *RUNX1-RUNX1T1* or *CBFβ-MYH11, PML-RARA* fusion transcript positive AML have a good response to chemotherapy. AML with t(9;11)(p22;q23) translocation or*MLLT3-MLL* fusion transcript, occurs in approximately 5-6% of all AML patients, more commonly in infants or in therapy-related AML patients. This abnormality is frequently associated with FAB classification M4 and M5 (acute myleomonocytic and monoblastic leukemias respectively). Three cytogenetically defined translocations were included in WHO 2008 classification: t(6;9)(p23;q34) with *DEK-NUP214* fusion gene, inv(3) (q21q26.2) or t(3;3)(q21;q26.2) with *RPN1-EVI1* fusion gene and t(1;22)(p13;q13) with *RBM15-MKL1* fusion gene.. These abnormalities occur most commonly in infants (Dohner; 2010).

The second, main category of the WHO 2008 classification, AML with myledysplasia-related changes (MDS-AML), integrates all AML cases with MDS is a group of acquired neoplastic disorders of the multipotent HSC characterized by increasing bone marrow failure of all three myeloid cell lines (Hoffbrand; 2001). AML patients are grouped as MDS-AML, if they had previously MDS or MDS/MPN and bone marrow or blood blast count above 20%, if they harbor MDS-related cytogenetic abnormalities (for example del (7q); del(5q); i(17q)) or if 50% or more of cells in 2 or more in myeloid lineages are dysplastic (Dohner; 2010). Therapy-related AML (t-AML) is the 3rd group of the WHO 2008 classification system. Patients with t-AML are known to have suffered of previous exposure to a cytotoxic agent or irradiation (Yin; 2010). The group of AML not otherwise

specified (AML, NOS) contains approximately 25-30% of AML cases that do not fulfill criteria for any of the AML other categories (Yin; 2010).

Myeloid sarcoma is caused by abnormal extramedullary proliferation of blasts of one or more of the myeloid lineages. It is mostly found in patients who were or recently are diagnosed with AML. Interestingly for AML patients who recently are in remission, a myeloid sarcoma is an evidence of a relapse. On the other hand for patients suffering from MDS, MDS/MPN it is an evidence of progression to AML (Vardiman; 2009).

The myeloid proliferations related to Down syndrome differ from other myeloid neoplasms in term of morphology, immunophenotype as well as clinical and molecular features with characteristic *GATA1* (GATA binding protein1, globin transcription factor 1) gene mutations (Vardiman; 2009).

The last group including blastic plasmacytoid dendritic cell neoplasm develops from a plasmacytoid dendritic cells (Vardiman; 2009). It is a very aggressive neoplasm. It manifests by solitary or multiple skin lesions (Vardiman; 2009).

Table 2. WHO classification of AML and related neoplasms including underlying genetic abnormalities.

I.	**Acute myeloid leukemia with recurrent genetic abnormalities**
	AML with t(15;17)(q22;q12); *PML-RARA* AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1* AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* AML with t(9;11)(p22;q23); *MLLT3-MLL* AML with t(6;9)(p23;q34); *DEK-NUP214* AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1* *Provisional entity: AML with mutated NPM1* *Provisional entity: AML with mutated CEBPA*
II.	**Acute myeloid leukemia with myelodysplasia-related changes**
III.	**Therapy-related myeloid neoplasms**
IV.	**Acute myeloid leukemia, not otherwise specified (NOS)**
	Acute myeloid leukemia with minimal differentiation Acute myeloid leukemia without maturation Acute myeloid leukemia with maturation Acute myelomonocytic leukemia Acute erythroid leukemia Pure erythroid leukemia Erythroluekmia, erythroid/myeloid Acute megakaryoblastic leukemia Acute basophylic leukemia Acute panmyelosis with myelofibrosis
V.	**Myeloid sarcoma**
VI.	**Myeloid proliferataions related to Down syndrome**
VII.	**Blastic plasmacytoid dendritic cell neoplasm**
VIII.	**Acute leukemias of ambiguous lineage**

Although WHO 2008 classification is more detailed, FAB classification may still be useful. FAB designation can be achieved easier and faster (morphological examination, cytochemical stains), and some of the groups of both classifications overlap, for example; FAB M3 and *PML-RARA* (Yin; 2010).

1.2 Treatment

Treatment of AML can be divided into *induction* and *consolidation* chemotherapy. The goal of induction therapy is to achieve a complete remission: the eradication of morphologically visible leukemia (immature blast cell less than 5 % in BM, and normal peripheral blood (PB) cell counts). The intensity of the induction treatment depends on patient´s age and health condition. More intense chemotherapy is usually given to younger patients (below 60 years old). The most commonly applied induction therapy protocol is

called ″7+3″, which includes two drugs, *cytarabine* infused consecutively for 7 days and an *anthracycline* derivate (*daunorubicin or idarubicin*) applied for 3 days. The induction therapy destroys not only the leukemia cells, but also most of the normal BM cells. At this stage patients are hospitalized and treated with antibiotics and are substituted with blood product transfusions. After the induction therapy, a small number of leukemia cells may still remain and the patient has to go under consolidation therapy in order to stay in remission (Hoffbrand; 2001).

Consolidation therapy is applied to eradicate any residual disease. Similarly to induction therapy, the applied consolidation chemotherapy depends on patient´s prognostic factors and condition. Patients with good prognosis usually receive 3-5 additional courses of chemotherapy consisting of high-dose cytarabine (*HiDAC*), while patients with adverse prognosis may undergo hematopoietic stem cell transplantation (HSCT). In case of HSCT, patients are treated first with total body irradiation and/or an intensive chemotherapy to destroy all BM cells. After that, they receive either allogeneic (from a donor) or autologous (patient´s own BM) HSCT to recover blood cell production. In case of allogeneic stem cell transplantation, stem cells are donated from another person, whose human leukocyte antigen (HLA) type is identical (matched) or close to identical (mismatched) to the patient´s type. The best donors are HLA identical siblings. If there is no donor available from the family, an HLA-type matched unrelated donor (MUD) is searched in the national and the international bone marrow registries. In autologous HSCT, the patient´s stem cells are first removed during remission and stored while the patient is further treated and then the stem cells are infused back to the patient. In case of autologous HSCT there is no risk either for the rejection of the transplanted hematopoietic stem cells or for the so called graft-versus-host disease (GVHD), but there is a higher risk for returning of the leukemic cells, i.e. relapse (Hoffbrand; 2001). The reason for developing GVHD is immunological incompatibility between donor and recipient. Donor´s immune cells, particularly T cells react against host tissues. The skin, the liver and the digestive system are most frequently affected. Although GVHD is a significant cause of mortality and morbidity in patients undergoing allogeneic HSCT, it appears to have some advantages, such as a phenomenon known as graft-versus-leukemia (GVL) effect. In case of GVL, the donor´s immune system helps to eradicate host´s leukemia. Relapse rate is known to be much lower in patients who developed GVHD (Hess; 2010, Hoffbrand; 2001). Older patients or those who are in worse conditions usually receive less aggressive consolidation therapy consisting of 1 or 2 cycles of higher dose chemotherapy (*cytarabine*) (Hoffbrand; 2001).

APL (FAB M3) with *PML-RARA* fusion gene, characterized by a high number of promyelocytes in peripheral blood and bone marrow, is treated differently. Drugs used in APL are called *differentiation agents* and their role is to signal HSC to differentiate into mature myeloid cells, in order to produce normal granulocytes. All-trans retinoic acid (ATRA) in combination with antracycline-based chemotherapy acts upon the PML-RARA (promyelocytic leukemia-retinoic acid receptor alpha) fusion protein by reversing the transcriptional blockage caused by the protein fusion.

After receiving treatment, one of the most important prognostic factors is the response to therapy. To better stratify the response to treatment, specific criteria were defined, such as complete remission, relapse, early death and resistant disease (Fig. 1).

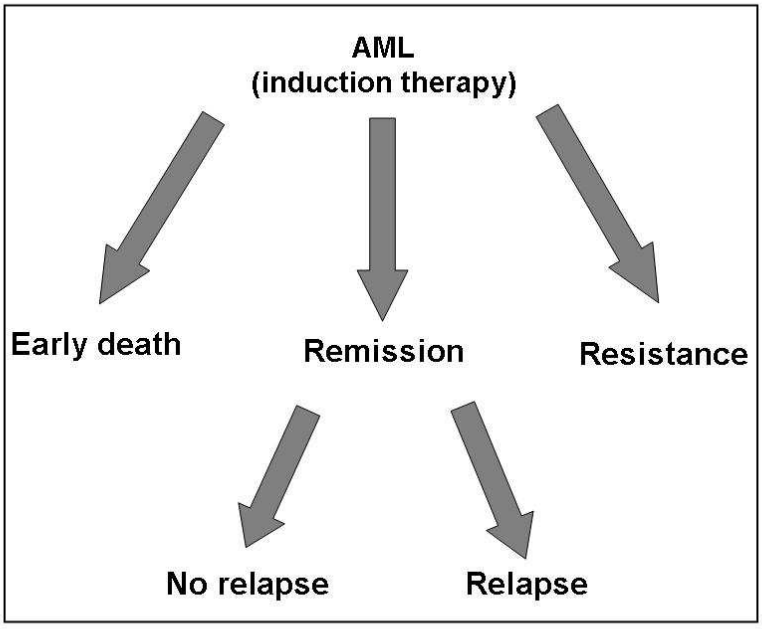

Figure 1. Treatment response in AML.
Early death occurs before the evaluation of the remission after treatment (usually before the 28th day after the initiation of the induction therapy). According to the morphological evaluation of the bone marrow on the 28th day, remission or therapy resistance is defined. After achieving remission, patient may either stay in remission or relapse.

Generally, complete remission is defined as the disappearance of signs and symptoms of a disease. In complete remission, the percentage of blasts in BM is less than 5%, the patient has normal values for absolute neutrophil (>1T/L) and platelet count (>100G/L) and no signs of extramedullary disease (Dohner; 2010). After remission patients may have relapse, which is described as a return of the disease after a period of improvement. In AML, relapse is defined as more than 5% of bone marrow blasts or as blasts in the peripheral blood or as the development of an extramedullary disease (Dohner; 2010). Resistant disease may be diagnosed only in patients surviving at least 28 days of therapy

and with evidence of persistent leukemia in the bone marrow or peripheral blood (Dohner; 2010). Early death happens if a patient dies within the first 28 days of therapy without the evaluation of remission or relapse (Dohner; 2010, Walter; 2011).

Different treatment outcome parameters are used to measure the effectiveness of therapies. Two most important factors indicating efficiency of therapy are duration of remission and overall survival (Fig. 2). Duration of remission is called disease free survival (DFS). It is defined only for patients who achieved complete remission. DFS is calculated as a time from the date of achievement of remission until the date of relapse. Patients who have not relapsed are censored on the day of the last examination. Patients, who died from any other cause, are considered as they never relapsed (competitive cause of failure) (Dohner; 2010). Overall survival (OS) is calculated from the date of diagnosis to the date of death from any cause or the date of the last follow up (the date, when the patient was last known to be alive) (Dohner; 2010).

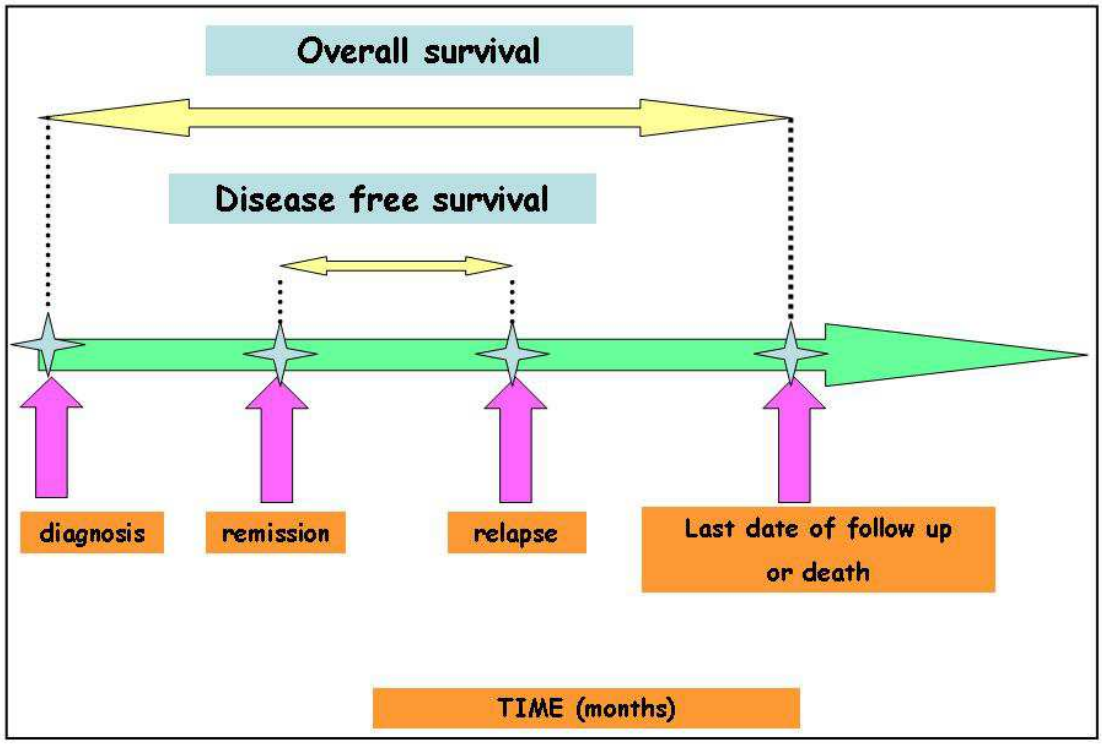

Figure 2. The description of treatment outcome as the measurement of the effectiveness of the therapy. Disease free survival (DFS) is defined as the duration of remission. The start point is the date of remission the ending point is the date when relapse or death from any cause occurs. Overall survival (OS) is defined as the duration of the patient´s life, starting from the date of diagnosis until the date of death or the last follow up.

1.3 Prognostic factors

Prognostic factors in AML can be divided into two main subgroups; patient-related and AML-related factors. Both account for a risk of premature death due to complications from

treatment, and death due to leukemia drug resistance or relapse and finally for the length of overall survival. As a result of new achievements in supportive care for AML patients, the risk of death in younger patients is now largely due to leukemia-related factors. In older patients, both patient- and leukemia-related factors still remain considerable obstacles to achieving better outcomes. Age is one of the most important host related factors. Patients older than 60 years are known to be associated with poorer outcomes (Appelbaum; 2006). There are other host-related factors besides age, including performance status, comorbidities and organ dysfunction (Kantarjian; 2006). The combination of age and performance status seems to be the most important host related factor in terms of prediction of 28-day mortality (Appelbaum; 2006) and in aftermath for the selection of patients for induction therapies (Yang and Schiffer; 2012).

Beside patient-related factors, a huge genetic diversity among acquired genetic background occurs in AML patients. Observed genetic mutations and altered gene expression help for the risk stratification (Dohner and Dohner; 2008, Mrozek; 2007, Schlenk; 2008). Detailed characterization of acquired genetic background in malignant disorders may help in establishing a targeted and a personalized treatment strategy for improving outcome.

1.3.1 Cytogenetic prognostic factors

Acquired cytogenetic abnormalities, found in approximately 55% of AML patients, are the most important leukemia-related prognostic factors for treatment outcome and survival (Mrozek; 2004, Schiffer; 1989, Yang and Schiffer; 2012). Acquired cytogenetic abnormalities are divided into 3 prognostic groups: favorable, adverse and intermediate.

The favorable cytogenetic risk group covers the structural changes of genes affecting the subunits of the core-binding factor (CBF): t(8;21), inv 16, t(16;16) in detail, or the *RARA* gene: t(15;17). Approximately 15% of AML patients belong to this group. These patients have a higher rate of CR and a longer duration of remission, which causes a longer overall survival (Dohner; 2010, Yang and Schiffer; 2012).

The adverse cytogenetic risk group includes: deletions of 5, 5q, 7, 7q, 17p, inv(3)(q21;q26), t(3;3)(q21;q26), rearrangements of (11q23) except t(9;11) and complex karyotypes (defined as either ≥three or ≥five abnormalities). Adverse karyotype occurs in approximately 25% of AML patients. These karyotypes are known to be associated with drug resistance (Dohner; 2010, Yang and Schiffer; 2012). 10% to 12% of AML patients harboring ≥3 or in some cases ≥5 chromosome abnormalities associate with the poorest

prognosis defined as complex karyotype. Chromosomal losses (5q, 17p, 7q) and gains (8q, 11q, 21q) are the most frequent patterns in complex karyotype (Dohner; 2010).

The intermediate cytogenetic risk group consists of other abnormalities, which do not belong either to the favorable or to adverse groups. Patients, who do not carry any cytogenetic abnormalities (nearly 50% of AML cases) have normal karyotype (NK-AML) requiring molecular markers for risk stratification, belong also into the intermediate cytogenetic risk group (Gilliland; 2004, Grimwade; 2010).

1.3.2 Molecular genetic prognostic factors

After cytogenetic abnormalities, acquired mutations and inherited polymorphic variants affecting the leukemic cells, are the next important factors for the selection of therapy and for stratification of treatment outcome and survival in AML patients.

1.3.2.1 Acquired alterations as risk factors

A common mutation occurring in about 25% of AML and 35% of NK-AML patients is the internal tandem duplications (ITD) of the *fms-like tyrosine kinase 3* (*FLT3*) gene encoding a class III receptor tyrosine kinase. *FLT3*-ITDs occur in the juxtamembrane domain (JMD) or in the tyrosine kinase domain 1 (TKD1), resulting in constitutive activation of this receptor (Breitenbuecher; 2009). The length of the insertions ranges from 3 to more than 400 base pairs (bp). It is always in frame and produces a functional protein (Blau; 2012, Kayser; 2009, Schnittger; 2012). Several previous studies attempted to characterize the impact of high mutant-to-total *FLT3*-ITD ratio (load) and the size of *FLT3*-ITD. While load was repeatedly shown to be associated with adverse prognosis, different conclusions emerged about how the length of the duplication influences the outcome (Blau; 2012, Gale; 2008, Kusec; 2006, Stirewalt; 2006).

The most frequent acquired, recurrent mutation identified in approximately 35% of AML and 55% of NK-AML patients is a 4 basepair long insertion in the *nucleophosmin 1* gene (*NPM1*). The mutation alters the nuclear localization signal of the NPM1 protein resulting in a dominant cytoplasmic localization (Falini; 2005, Falini; 2007). *NPM1* mutations can be observed from the pre-leukemic hematopoietic stem cell throughout the whole disease course and also at relapse (Becker; 2010, Falini; 2011). AML patients harboring this mutation have a better prognosis. The reason for this is enhanced sensitivity to chemotherapy and in turn higher CR rates and longer survival (Yang and Schiffer;

2012). Even older patients, harboring *NPM1* mutation were reported to have a better prognosis (Becker; 2010).

A new type of recently identified, acquired genetic alteration in AML affects mutational hotspots of the isocitrate dehydrogenase 1 and 2 (*IDH1*codon 132 *and IDH2* codons 140 and 172) genes. Under normal circumstances, *IDH* enzymes catalyze the conversion of isocitrate to α–ketoglutarate (alpha-KG). The presence of *IDH* mutant enzymes results in aberrant production of 2-hydroxyglutarate (2HG), a structural analogue of alpha-KG and competitive inhibitor of alpha-KG-dependent enzymes. The production of 2HG is a common neomorphic activity of all *IDH1* and 2 mutant enzymes resulting in the block of alpha-KG dependent enzymes such as tet methylcytosine dioxygenases 1 and 2 (*TET1 and 2*), or histone demethylases causing aberrant DNA and histone methylation, altered gene expression profiles and consecutively impaired stem cell differentiations (Figueroa; 2010a, Lu; 2012, Xu; 2011).

1.3.2.2 Inherited variants as risk factors

The clinical manifestation and the treatment outcome of AML depend not only on the acquired mutations in the malignant clone, but also on the inherited genetic variations of the individual patient affecting e.g. pharmacokinetics, DNA-repair, disease phenotype or treatment outcome, signal transduction/epigenetic pathways, drug metabolism or transport.

The ABC-transporter genes encode evolutionary conserved transmembrane proteins with a common ATP-binding cassette (ABC) motif, consisting of 49 members. ABC genes are divided into 7 groups (ABCA to ABCG). Some ABC proteins, including; P-gp (ABCB1 or MDR1), ABCC1 (MRP1), ABCA3 and ABCG2 (BCRP) are known to mediate multidrug-resistance of leukemic cells.

The ATP-binding cassette, sub-family G, member 2 (*ABCG2*) multidrug transporter is preferentially expressed in pharmacological barriers (liver, kidney, placenta, blood-brain barrier). This protein modulates the absorption, metabolism and toxicity of numerous drugs and xenobiotics, and causes multidrug resistance in cancer (Doyle; 1998, Sarkadi; 2006). Interestingly, *ABCG2* was also found to be expressed in various stem cells (including hematopoietic and leukemic stem cells in AML) (Ding; 2010). The exact physiological function of ATP-binding cassette (ABC) transporters such as P-glycoprotein (encoded by the *ABCB1* gene) and breast cancer resistance protein (older name: BCRP, newer name: *ABCG2*) on the stem cell plasma membrane is not known, but they are generally presumed to play a protective role against a wide array of toxic substances. ABC-transporters may

also be responsible for the efflux of small lipophilic substrates, such as steroids, and their expression may play a regulatory role in inducing growth, differentiation or apoptosis.

A number of SNPs causing mis-trafficking and early degradation were identified in the coding region of the *ABCG2* gene. To date, among the most studied variants the following two nonsynonymous SNPs are represented: V12M (c.34G>A), where valine is replaced by methionine at codon 12 in exon 2 and Q141K (c.421C>A), where glutamine is replaced with lysine at codon 141 in exon 5. V12M has not yet been associated with any deviation in protein expression or function (Morisaki; 2005). On the other hand, Q141K, with a variable allele frequency between 5-30% in various ethnic groups (Cervenak; 2006), but preferentially occurring in Asian populations (Kobayashi; 2005), was found to decrease protein expression in vitro (Kobayashi; 2005, Morisaki; 2005), and to reduce drug efflux (Morisaki; 2005) and reduced ATPase activity (Mizuarai; 2004, Morisaki; 2005). Still, a lower expression level of the ABCG2 Q141K variant was not been confirmed at physiologically relevant sites, given the difficulties in obtaining and processing human tissues.

It has been shown earlier that the erythrocyte membrane contains functional ABCG2 protein (de Wolf; 2007, Leimanis and Georges; 2007, Maliepaard; 2001, Zhou; 2005). In 2012, two papers have been published, linking the high frequency blood group *Jun* to the ABCG2 protein, showing that *Jun*- individuals have no ABCG2 expression in their red cell membranes. These individuals carried mutations in their *ABCG2* gene on both alleles, resulting in early termination of transcription, while no apparent disease conditions were observed (Saison; 2012, Zelinski; 2012).

2. AIMS

Impact of acquired mutations (FLT3, NPM1, IDH1/2) in AML

Recent studies suggest that personalized medicine is an important tool in order to choose the most optimal therapy for cancer patients. Detailed characterization of acquired and inherited genetic background in malignant disorders, such as AML, is essential in risk stratification and in selection of the most optimal therapy, for each patient. Our aims were the following in the context of acquired mutations:

1. To set up and optimize a PCR followed by a capillary electrophoresis in order to calculate the frequencies of *FLT3* internal tandem duplications and *NPM1* mutations in our AML cohort.
2. To study the association between *FLT3*-ITD load, size and clinical characteristics or treatment outcome in AML.
3. To set up and compare three different detection methods (allele specific PCR, high resolution melting and sequencing) in order to calculate the frequencies of *IDH1/2* mutations in AML.
4. To verify the clinical and the prognostic relevance of different *IDH1/2* mutations in AML.

ABCG2 genetic variations in healthy individuals and in AML patients

Inherited genetic variations have also been found to play an important role in the clinical manifestation and the treatment outcome of AML patients. They account for risk stratification and choice of therapy in order to achieve the best outcome. In connection, our aims were the following:

1. To develop an antibody-based quantitative flow cytometric assay for the determination of membrane proteins including ABCG2 on red blood cell (RBC) membranes.
2. To test the effect of common *ABCG2* polymorphic variants (V12M and Q141K) on ABCG2 protein expression on RBC membrane in healthy individuals.
3. To investigate the effect of genetic variants on disease susceptibility, clinical manifestation and treatment outcome in AML patients.

3. METHODS

3.1 Subjects

The AML cohort consisted of 389 consecutive patients [183 males/206 females; median age: 50 years (range: 16-93)]. The patients were diagnosed and treated at the Department of Hematology and Stem Cell Transplantation, St. István and St. László Hospital (formerly National Medical Center) between 2001 and 2009. The minimal follow-up was 12 months (maximum: 107 months). Clinical data (age, sex, etiology of AML and morphology according to French-American-British classification of [FAB]) and laboratory data (white blood cell count [WBC], platelets [PLT]) were collected retrospectively. Germline *ABCG2* SNPs were investigated in all 389 cases, while *FLT3*-ITD and *IDH1/2* mutations were tested only in cases, where DNA sampling was available at the time point of diagnosis or relapse (n=324 younger patients for *FLT3*-ITD, n=376 for *IDH1/2*). Treatment outcome parameters were investigated only in younger patients (<60 years at diagnosis), who were treated with curative intention (n=324; 150 males/174 females; median age: 47 years (range: 16-60). Complete remission, early death (less than 28 days survival after the start of therapy), resistant disease, disease-free survival (DFS) and overall survival (OS) were defined according to recommended criteria (Dohner; 2010). Cytogenetic abnormalities, based on at least 20 cells in metaphase, were described according to the International System for Human Cytogenetic Nomenclature (ISCN 2005). Karyotyping was performed in the Cytogenetic Laboratory of St. István and St. László Hospital.

Among healthy volunteers, 47 unrelated individuals and 14 family members of two probands selected from the volunteers were analyzed for ABCG2 expression by flow cytometry. Further 155 healthy volunteers were also genotyped for ABCG2 SNPs. Patients and healthy volunteers signed informed consents in agreement with the Regional Ethics Committee approval.

3.2 DNA and RNA isolation

Fresh peripheral blood or bone marrow was collected in tubes containing EDTA, as an anticoagulant. Frozen samples were stored at -20°C. DNA was isolated from fresh or frozen peripheral blood or bone marrow. DNA was extracted with Puregene Gentra Isolation Kit (Gentra Puregene Blood Kit, 1000 ml, catalogue number: 158389) according to the manufacturers' instructions. Isolated DNA was stored at -20°C.

Total cellular RNA was isolated at the time of diagnosis, from the bone marrow or peripheral blood, anticoagulated with EDTA or Na-citrate. RNA was extracted using Trizol reagent (Invitrogen, catalog No. 15596018), followed by reverse transcription by the High Capacity cDNA RT kit with RNase Inhibitor (Life Technologies, Catalog No. 4374966) reagent kit. For reverse transcription approximately 3 µg of RNA was used. Reverse transcription was performed in the following conditions: 25ºC for 10 min, 37 ºC for 120 min and 85ºC for 5 min. cDNA was stored at -20°C.

3.3 Molecular genetic methods

Detection of *FLT3*-ITD, *NPM1*, *IDH1/2 mutations* were performed from genomic DNA isolated from bone marrow or peripheral blood samples at the time of diagnosis. *ABCG2* expression level measurements were also performed at the time of diagnosis, but from mRNA isolated from bone marrow. *ABCG2* SNPs testing was performed from genomic DNA isolated from bone marrow or peripheral blood samples, but not always at the time of diagnosis.

3.3.1 Fragment analysis

In fragment analysis, one of primers is fluorescently labeled, which is incorporated in the PCR product during the reaction. The fluorescently labeled PCR product is separated by capillary electrophoresis allowing the precise sizing (in base pair resolution) and the relative densitometric quantification of the mutant and the wild type alleles. Fluorescently labeled primers for *FLT3*-ITD (Kottaridis; 2001) and *NPM1* mutation detection (Thiede; 2006) were adapted from previous publications (Table S1). PCR for *FLT3*-ITD was performed in a final volume of 10 µl and for *NPM1* in 20 µl. In both cases we used 2x PCR Master Mix (Promega, M7505) and 50ng of DNA. All primer concentrations were 0.5 µM. PCR reaction was carried out in 35 cycles (95°C 30 s, 55°C 40 s, 72°C 60 s). PCR products were analyzed first on 3% agarose gels followed by capillary electrophoresis by Beckman Coulter CEQ 8000 Genetic Analyzer with Genescan Analysis Before the capillary electrophoresis, all samples were mixed with GenomeLab^{TM} Sample Loading Solution (SLS) (Beckman, M209093) and with GenomLab^{TM} DNA Size Standard Kit-600 (Beckman, M309165). SLS prevents DNA from re-annealing after denaturation at 95°C. It is important to use single stranded DNA, because it can be easier separated in the GenomeLab^{TM} Separation Gel-LPA I (catalog no. M304363). Size marker consists of DNA

fragments of known length labeled with specific dye. All capillaries are filled with a special polymer, which enables DNA to move toward the positively charged anode. In the end of the capillary, there is so called detection window, where the laser is placed. Laser excites the dye attached to the primer and incorporated in the PCR product and induces a fluorescence signal, which next is converted to an electrical signal. In order to evaluate the signal two units have to be considered: the fragment size of the peak in base pairs (bp) along x-axis and the fluorescence signal intensity described with peak height or area under the curve values along the y-axis. As peak height values were available in more cases, peak height values were used for the calculation of ITD load. The *FLT3*-ITD load was quantified as the ratio of the peak height of the *FLT3*-mutant allele divided by the sum of the mutant and the wild type peak heights (ITD/ITD+WT).

3.3.2 High Resolution Melting (HRM)

HRM is a fluorescence based method for rapid mutation screening of a region with a variant which exact position or type is not known. The method requires only the usual unlabeled primers and double-stranded saturating DNA dye. HRM differs from a regular SNP genotyping in three aspects; it uses special brighter dsDNA saturating dye that fluoresce only in the presence of dsDNA, different fluorescent algorithms and plots are applied and the most importantly more data points are collected than with a standard melt curves. When the samples are heated, the DNA denatures and the fluorescent color fades away as the double stranded DNA separates, generating a melting curve. Because different genetic sequences melt at slightly different rates, they can be detected using these curves. Even a single base change will cause differences in the melting curve. The process can be used for specific genotyping, comparing sequence identity between two DNA samples, and scanning for any sequence variant between two primers. After amplification, melting curve analysis is performed by gradually heating the samples from 40 to 95°C with continuous fluorescence monitoring. During the process differences in melting curves are detected in the real-time PCR machine.

HRM for *IDH1/2* was performed in parallel with AS-PCR in patients with normal karyotype and in patients with positive AS-PCR screen. HRM analysis was carried out using LightCycler 480 System (Roche Diagnostics, Basel, Switzerland), in a reaction volume of 10 µl, including 5 µl 2x HRM Master Mix (Roche, 4909631001) containing Resolight dye as saturating dsDNA dye, supplemented by 25 mM $MgCl_2$ to a final $MgCl_2$ concentration of 1 mM. All *IDH1*-HRM and the reverse *IDH2*-HRM primers were adopted

from (Tefferi; 2010). *IDH2*-HRM140Q-F, *IDH2*-HRM172K-F were designed in house (Table S2). For amplification, we used 50 ng of DNA. Primer concentrations were 0.24 µM. Reaction was carried in 35 cycles (95°C 600 s, 95°C 30 s, 60°C 40 s, 72°C 60 s), with a ramp rate of 4.4°C/s. After amplification, melting curve analysis was performed. During this process, double stranded dsDNA becomes denatured. The melting temperature (Tm) of the PCR products depends on the sequence of DNA. If there is a sequence variant (even a single nucleotide mismatch) in the PCR product, it alters Tm compared to the wild type PCR product. Melting was carried out in 1 cycle (95°C 60 s, 40°C 60 s and then gradually heating to 95°C with a ramp rate of 4.4°C/s). The decline of fluorescence was continuously monitored. Variant genotypes were distinguished from the wild-type genotype by an altered amplicon melting curve shape.

3.3.3 Allele specific PCR

Allele-specific polymerase chain reaction (AS-PCR) was used to detect point mutations affecting *IDH1/2* genes. In this method, one of the primers contains a 1 or 2 basepair long, 3′ mismatch sequence compared to the wild-type DNA template, and it is refractory to primer extension by *Taq* DNA polymerase. The mismatched primer allows the formation of allele-specific PCR products of different lengths, which may be separated by agarose gel electrophoresis in the presence of the investigated point mutation (Ugozzoli and Wallace; 1992). AS-PCR was used as a primary screening method in all AML patients. AS-PCR for *IDH1* R132 codon mutations was adapted from Chou (Chou; 2010b). Seven primers were used including 2 controls (*IDH1*-Fcont and *IDH1*-Rcont) and 5 different mutation specific primers (*IDH1*-R132C, *IDH1*-R132H, *IDH1*-R132G, *IDH1*-R132L, *IDH1*-R132S). A similar single-tube, multiplex AS-PCR method was developed in house, for the simultaneous detection of *IDH2* R140 and R172 mutations. To detect R140Q and R172K mutations, four primers were designed including two control (*IDH2*-Fcont and *IDH2*-Rcont), an *IDH2*-R140Q specific (*IDH2*-R140Q) and an *IDH2*-R172K specific primer (*IDH2*-R172K) (Table S3).

PCR for *IDH1/2* was performed in final volume of 20 µl, including 2x PCR Master Mix (Sigma-Aldrich, P4600-100RXN), 50 ng of DNA. *IDH1*-Fcont and *IDH2*-Fcont primer concentrations were 0.15 µM. *IDH1*-Rcont, *IDH2*-Rcont and mutation specific primer concentrations were 0.5 µM. PCR was carried out in 35 cycles (95°C 30 s, 60°C 40 s, 72°C 60 s). PCR products were analyzed on 3 % agarose gels (Table S3).

3.3.4 Sequencing

The classical chain-termination (Sanger) sequencing requires a DNA template, a DNA primer, a DNA polymerase, normal deoxynucleoside-triphosphates (dNTPs), and modified dideoxynucleotides (ddNTPs) that terminate DNA strand elongation. In order to test four ddNTPs, the different ddNTPs are labeled with four different fluorescent dyes. Afterward the DNA fragments are heat denatured and separated by size using capillary electrophoresis.

In cases of *IDH1,* sequencing was performed in order to determine the exact amino acid substitution in codon 132, previously detected by AS-PCR and HRM. In case of *IDH2* sequencing was performed in case of contradictory results detected by AS-PCR and HRM. To generate a PCR product for sequencing, the formerly mentioned (Table S3) primers were used: *IDH1*-Fcont and *IDH1*-Rcont for *IDH1;* and *IDH2*-Fcont and *IDH2*-Rcont for *IDH2*. PCR reaction was carried out in 35 cycles (95°C 30 s, 60°C 40 s, 72°C 60 s). PCR was performed in a final volume of 20 µl, including 2x PCR Master Mix (Sigma-Aldrich, P4600-100RXN) and 50 ng of DNA. Primer concentrations were 0.5 µM. PCR products were first analyzed on 3% agarose gels and afterward cleaned using the ExoSap Kit (Amersham, US78201). Cleaning conditions were the following: 37°C for 30 min and 80°C for 15 min. Dye terminator cycle sequencing (DTCS) Quick Start Kit (Beckman BECK-608120) was applied for sequencing reaction. Sequencing was performed in 30 cycles (95°C 20 s, 55°C 20 s, 60°C 240 s). Sequences were analyzed by Beckman Coulter CEQ 8000 Genetic Analyzer.

For the *ABCG2* gene, the coding region (exons 2-16) and the exon-intron boundaries were sequenced in order to identify novel mutations. For this purpose, 27 primers were adopted (Zelinski; 2012), see (Table S6). PCR reaction and cleaning process of the generated PCR products were the same as for *IDH1/2*. The Big Dye Terminator Kit (catalog no. 4337455) was applied for sequencing reaction. Sequencing was performed in 25 cycles (95°C 30 s, 60°C 240 s). Sequencing products were analyzed using Applied Biosystems 310 Genetic Analyzer (Life Technologies, Carlsbad, USA).

3.3.5 Quantitative PCR (QPCR)

Quantitative polymerase chain reaction (QPCR), also called real-time PCR is used to amplify and quantify DNA or RNA. The amplified PCR product is detected "real time" in each cycle. The amplification is monitored by a sequence-specific oligonucleotide probe.

The so called TaqMan-probe is labeled with two fluorescent dyes (a reporter and a quencher dye) which permits detection only after hybridization of the probe with its complementary sequence. In our study, QPCR was used to estimate *ABCG2* mRNA expression levels in the bone marrow of a subset of AML patients at diagnosis (n=81).

ABCG2 expression was normalized to *ABL1* as a reference gene. PCR (for both *ABL1* and *ABCG2* genes) was performed in a plate format in a reaction volume of 10 µl, containing 5 µl Light Cycler® 480 Probes Master 2x concentrated (Roche, 04 707 494 001). *ABL1* primers and TaqMan probe were adopted from an international recommendation for the best reference genes applicable in QPCR (Beillard; 2003) (Table S4). An "on-demand" TaqMan assay for *ABCG2* (Life Technologies, catalog no. Hs01053790_m1) was applied. Cycling conditions were the following: initial denaturation at 95°C for 600 s, followed by 45 cycles of denaturation 95°C for 10 s with a ramp rate of 4.4°C/s, annealing and extension at 60°C for 30 s with a ramp rate of 2.2°C/s. The expression level of *ABCG2* mRNA was calculated according to the crossing points (Cp) of *ABCG2* and compared to the crossing points of the reference gene, *ABL1* measured in the same cDNA sample, using the ΔCp method. Crossing point is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold. The Cp value is inversely proportional to the original amount of the gene of interest. ΔCp method calculates the relative expression ratio, calculated by the comparison of the Cp values of a target gene to the Cp of a reference gene.

3.3.6 Genotyping of *ABCG2* SNPs

Genotyping was performed by melting curve analysis with the application of internally labeled, with a fluorescence dye at a thymine (T) nucleotide hybridization probes (a donor and acceptor), added together with the amplification primers. Donor and acceptor were placed in close proximity and the emission spectrum of one overlapped significantly with the excitation spectrum of the other. This is called a fluorescence resonance energy transfer (FRET). During FRET, the donor fluorophor excited by a light source, transferred its energy to an acceptor, acceptor fluorophor gave a signal, detected by the machine. The fluorescence was changing depending on mismatches in the sequence.

The most common SNPs in *ABCG2* [V12M (c.34G>A, p.12Val>Met in exon 2, SNP database ID: rs2231137) and Q141K (c.421C>A, p.141ln>Lys in exon 5, SNP database ID: rs2231142)] were genotyped using the LightCycler480 (Roche Diagnostics, Basel, Switzerland) allelic discrimination system as described previously in detail (Fischer;

2007). PCR was performed in a plate format in a reaction volume of 10 µl, including 5 µl 2x PCR Master Mix (Promega, M7505), supplemented by 0.7 U *Taq* DNA polymerase (Finnzyme, Espoo, Finnland, catalogue # F-501L), and by $MgCl_2$ to the final concentration of 2 mM. In both cases (V12M and Q141K) two primers were used a forward primer (LCF) and reverse (LCR), and two hybridization probes anchor (ANC) and sensor (SENS) (Table S5). To improve the amplification efficiency of the strand complementary to the hybridization probes, an asymmetric PCR was used for amplification with 5:1.5 LCF:LCR and 1:10 LCF:LCR amounts for V12M and Q141K respectively (Szilvasi; 2005). We used 50 ng of DNA. Cycling conditions were the following: initial denaturation at 95°C for 90s, followed by 70 cycles of denaturation 95°C for 20 s, annealing at 50°C for 30 s, and extension at 72°C for 45 s, with a ramping rate of 4.4°C/s. After amplification, melting curve analysis was performed by first heating the samples up to 95°C for 30 s and next cooling them down to 32°C for 40 s, then gradually heating them up to 75°C (V12M) or 85°C (Q141K) with a ramp rate of 0.04°C/s. The decline of fluorescence was continuously monitored. Melting curves were converted to melting peaks with wild type and variant alleles showing distinct melting points.

3.4 Flow cytometry (FACS)

Flow cytometry is a laser based method used in cell biology. It is applied for counting cells. It investigates cells by passing them in fluid form by special detector. It is very fast and convenient method for physical and chemical characterization of cells.

FACS analysis was performed in Membrane Research Group of the Hungarian Academy of Sciences, Semmelweis University, Budapest, Hungary by György Várady and Adrienn Németh (group leader Balázs Sarkadi). The determination of ABCG2 protein expression level on red blood cells (RBC) was performed in 61 healthy volunteers (47 unrelated individuals and 14 family members of two probands selected from the volunteers). For this purpose 25 ul of freshly drawn human peripheral blood and three antibodies; including BXP21 and BXP34 recognizing the ABCG2 protein on an intracellular epitope and 5D3 recognizing the same protein on an extracellular epitope were used. Intact red cells and erythrocyte ghost were gated based on the forward scatter (FSC) and side scatter (SSC) parameters. Both fractions were analyzed for antibody staining by a FACSCalibur flow cytometer (excitation wavelength: 488 nm (Argon ion laser) emission filters: 585/42 nm for PE, 530/30 for FITC). For ABCG2 expression controls, K562

(human immortalized myelogenous leukemia cell line) cells retrovirally transduced to express ABCG2 were used, as described previously (Hegedus; 2009).

3.5 Statistical analysis

Continuous variables are presented as median and range. The Mann-Whitney or the Kruskal-Wallis tests were used to compare continuous variables in subgroups according to *FLT3*-ITD mutation status, different ITD load and size and *IDH* mutation status. Fischer's exact test and the $\chi 2$ tests were performed to compare dichotomous variables. Genotype and allele frequencies with 95% confidence interval (AF ± 95%CI) are presented. Estimated haplotype frequencies (EHF) were calculated by the web-based SNPStats web tool (Sole; 2006). The log-rank test was used to compare DFS and OS between groups separated by ITD load and size *or IDH* mutation status or *ABCG2* genotypes. A Cox proportional hazards model was computed for multivariate analysis of OS and DFS with the calculation of hazard ratio (HR) and 95% confidence interval (95%CI). The statistics were performed using Statistical Package for the Social Science (SPSS) version 13.0.

4. RESULTS

4.1 FLT3 internal tandem duplication in AML

4.1.1 Determination of *FLT3*-ITD and *NPM1*

To characterize the frequencies of *FLT3*-ITD and *NPM1* mutations, we screened 324 AML patients. *FLT3-ITD* and *NPM1* mutational status was defined according to the detected fluorescently labeled PCR product on capillary electrophoresis fragment analysis (Fig. 3&4 respectively). Wild type PCR products were 329 bp and 289 bp long for *FLT3*-ITD (Fig. 3A) and *NPM1* (Fig. 4A) respectively. The presence of ITD gives a longer PCR product, but the size depends on the length of the insertion (Fig. 3C&D). *NPM1*^{pos} is always a 4 bp long insertion, which gives 293bp long PCR product (Fig. 4B&C). *FLT3-ITD* mutations were detected in 25.3% (82/324) of AML cases and *NPM1* mutations in 29.9% (97/324). In 6.8% (22/324) cases more ITDs are present at the same time (Fig. 3B).

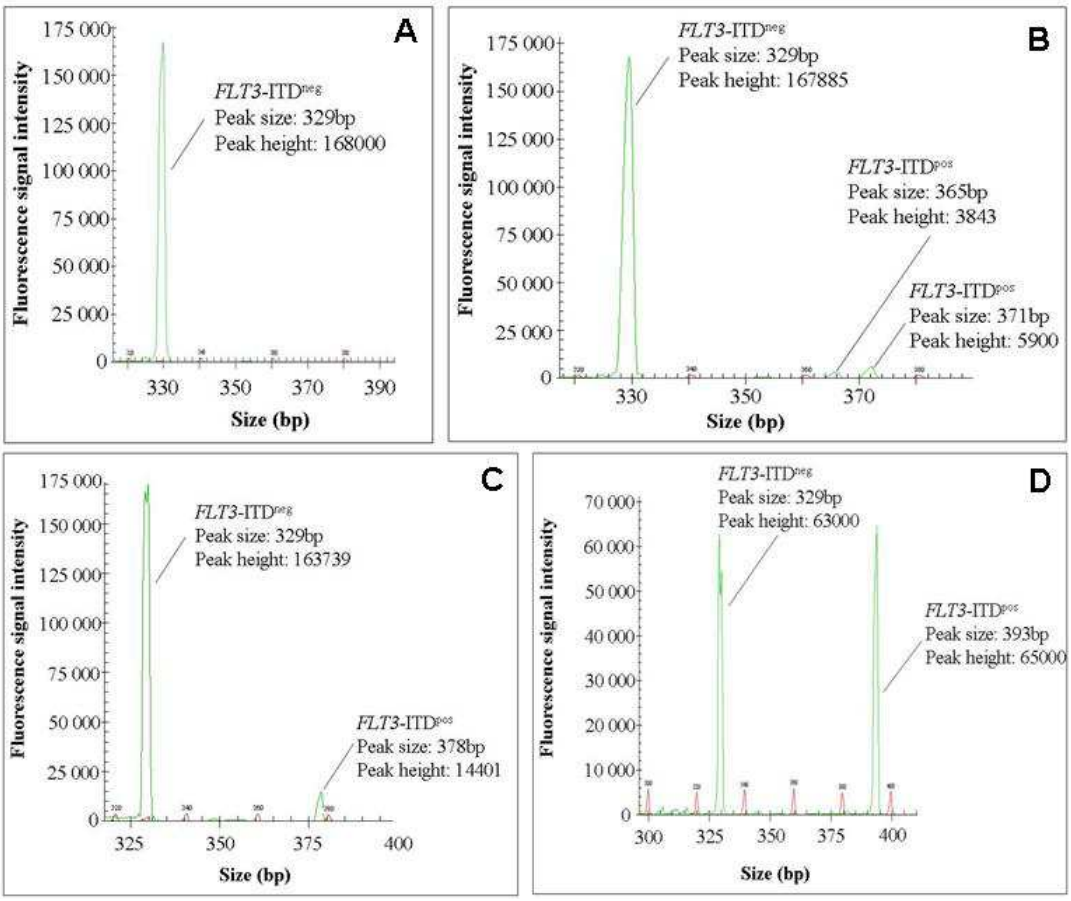

Figure 3. Fragment analysis of FLT3-ITD mutation. Red peaks represent marker, green ones PCR product. The 329bp long PCR product illustrates wild type allele, any other PCR product longer than 329bp represents a mutant allele.

A) FLT3-ITD negative:, only wild type allele is visible with the peak size of 329bp and peak height of 168000.

B) FLT3-ITD positive: three peaks are visible: wild type allele (size: 329bp, height: 167885) and mutant alleles (size: 365bp and 371bp, height: 3843 and 5900 respectively). Mutant allele load=mutant FLT3-ITD/ (mutantFLT3-ITD+wild type FLT3-ITD) = 2% and 3% respectively.

C) FLT3-ITD positive: two peaks are visible: wild type allele (size: 329bp, height: 163739) and a mutant allele (size: 378bp, height: 14401). Mutant allele load =8%.

D) FLT3-ITD positive: two peaks are visible: wild type allele (size: 329bp, height: 63000) and a mutant allele (size: 393bp, height: 65000). Mutant allele load =50%.

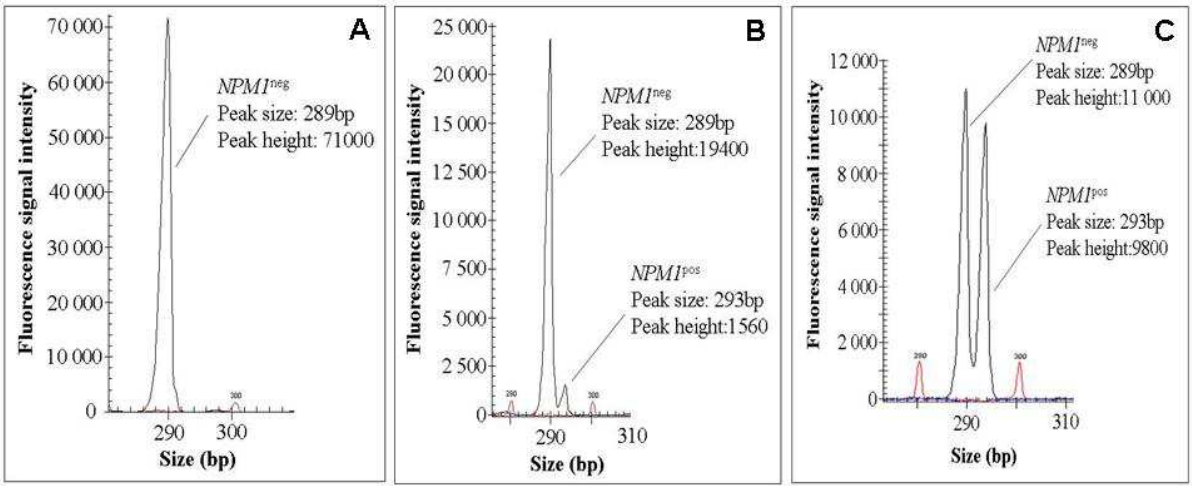

Figure 4. Fragment analyses of NPM1 mutations.
Red peaks represent size marker, black ones PCR product. 289bp long PCR product illustrates wild type allele and 293bp long mutant allele.
A) NPM1 negative sample: only the wild type allele is visible with the peak size of 289bp and peak height of 71000.
B) NPM1 positive sample: two peaks are visible: wild type allele (size: 289bp, height: 19400) and a mutant allele (size: 293bp, height: 1560). Mutant allele load =height of the mutant NPM1/ (height of the mutant NPM1+height of the wild type NPM1) = 7%.
C) NPM1 positive sample, two peaks are visible: wild type allele (size: 289bp, height: 11000) and a mutant allele (size: 293bp, height: 9800). Mutant allele load=47%.

4.1.2 Clinical characteristics according to *FLT3*-ITD mutation status

4.1.2.1 Associations between clinical characteristics and the presence of *FLT3*-ITD

The presence of *FLT3*-ITD mutation showed no association with age or sex. The etiology of AML was more frequently *de novo* in *FLT3*-ITD^{pos} patients (83.9% compared to 56.1% in *FLT3*-ITD^{neg} patients; p<0.0001). M5 morphology subgroup was more prevalent among *FLT3*-ITD^{pos} patients (33.8% vs. 14.6% in *FLT3*-ITD^{neg} cases, p=0.0004), while M1 was less common (12.2% vs. 28.9% in *FLT3*-ITD^{neg} cases, p=0.003). Higher WBC (*FLT3*-ITD^{pos}: 60 G/L vs. *FLT3*-ITD^{neg}: 6.35 G/L, p=0.0001) was observed among *FLT3*-ITD^{pos} patients. The *FLT3*-ITD^{pos} patient group had intermediate risk karyotype more frequently (*FLT3*-ITD^{pos}: 85.5%, vs. *FLT3*-ITD^{neg}: 50.3%; p<0.0001) and only 7.9% had favorable and 6.6% adverse karyotype vs. *FLT3*-ITD^{neg} 20.2% and 29.4% respectively (p=0.01, p<0.0001). *FLT3*-ITD associated with *NPM1* mutations (*FLT3*-ITD^{pos} 58.7% vs. *FLT3*-ITD^{neg} 16.4%; p<0.0001). Pre-treatment clinical, cytogenetic and molecular characteristics according to the *FLT3*-ITD mutation status are presented in Table 3.

Table 3. Pre-treatment, clinical and molecular characteristics according to *FLT3*-ITD mutation status.

Characteristics	***FLT3*-ITD^{neg} (n=255, 78.7%)**		***FLT3*-ITD^{pos} (n=69, 21.3%)**		**p**
	number/ median	**%/range**	**number/ median**	**%/range**	
Age (median, range)	48	(16-60)	45	(18-60)	0.03
Sex (male/ female; %)	122/133	47.8/52.2	28/41	40.6/59.4	0.34
Type of AML (n, %)					
De novo	153/253	60.5	56/69	81.2	**<0.0001**
MDS-AML	80/253	31.6	11/69	15.9	**0.01**
t-AML	20/253	7.9	2/69	2.9	0.18
FAB (n, %)					
M0	8/238	3.4	1/65	1.5	0.69
M1	75/238	31.5	9/65	13.8	**0.005**
M2	31/238	13.0	7/65	10.8	0.83
M3	23/238	9.7	4/65	6.2	0.47
M4	61/238	25.6	22/65	33.8	0.21
M5	34/238	14.3	22/65	33.8	**0.0009**
M6	3/238	1.3	0/65	0.0	1
M7	3/238	1.3	0/65	0.0	1
Laboratory data, median (range)					
WBC at diagnosis G/L (n=309)	6.45	(.090-368)	44.3	(.3-334)	**<0.0001**
PLT at diagnosis G/L (n=230)	42	(5-684)	45	(5-263)	0.80
Cytogenetics (n, %)					
Favorable	51/244	20.9	5/67	7.5	**0.01**
Intermediate	125/244	51.2	58/67	86.6	**<0.0001**
Normal	92/244	37.7	42/67	62.7	**0.0004**
Adverse	68/244	27.9	4/67	6.0	**<0.0001**
Mutations (n, %)					
NPM1^{pos}	44/254	17.3	37/67	55.2	**<0.0001**

Significant p values are shown in bold.

Abbreviations: AML: acute myeloid leukemia; FAB: morphology according to French-American British classification; *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication; MDS-AML: AML evolving from myelodysplastic syndrome; *NPM1* ^{pos}: nucleophosmin 1 positive; PLT: platelet count at diagnosis; t-AML: therapy-related AML; WBC: white blood cell count at diagnosis.

4.1.2.2 Clinical characteristics according to *FLT3*-ITD load

The median *FLT3*-ITD load was 26% (range: 1%-81%; 25th-75th percentiles: 11%-40%). Different thresholds (25th percentile, median and 75th percentile) were systematically used in the analyses for the evaluation of the prognostic impact of ITD burden. Comparisons applying the threshold of 50% mutant/total ratio indicative for the loss of the wild type allele (uniparental disomy, UPD) in the leukemic clone were also performed. No significant differences regarding treatment outcome were observed using the thresholds at 11%, 26% or 40%. Using 50% as a cut-off, clinical characteristics at presentation (sex, etiology of AML, FAB, PLT, cytogenetics and *NPM*1), were not different between *FLT3*-ITD^{pos} individuals with low ITD load (*FLT3*-ITD$^{<50\%}$; n=56) vs. high ITD load (*FLT3*-ITD$^{>50\%}$; n=15, data not shown). Interestingly, patients with *FLT3*-ITD$^{>50\%}$ were younger

(median: 41, range: 20-60 years) compared to the *FLT3*-ITD$^{<50\%}$ (median: 50; range: 19-83 years; p=0.011) and to the *FLT3*-ITD^{neg} (p=0.016).

4.1.2.3 Clinical characteristics according to *FLT3*-ITD size

The size of *FLT3*-ITD varied from 6 to 210 base pair (bp) (median 39bp; 25th-75th percentile: 24-60bp). 22/81 patients (27.2%) displayed more than one mutant amplicons (size calling was possible in 81/82 *FT3*-ITD positive cases). In case of double mutant, the predominant variant (with the highest mutational load) was chosen for size analyzes.

To analyze possible associations between *FLT3*-ITD mutation sizes and outcome, we initially divided the *FLT3*-ITD^{pos} patient group into two subgroups with the following cut-offs: median (39 bp), 25th percentile (24 bp), 75th percentile (60 bp) and the most relevant values (33 and 48 bp) from the literature (Bhatt and Abdel-Wahab; 2013). After clinical outcome analyzes (Kaplan-Meier survival curve, relapse rate comparisons), the most striking differences were found using the cut off of 48 bp (*FLT3*-ITD$^{<48bp}$; n=44, *FLT3*-ITD$^{>48bp}$; n=37), thus this cut-off was used for further comparisons of the dichotomous groups. No significant differences were observed between these two groups regarding clinical data (sex, etiology of AML, FAB, laboratory data, cytogenetics and other mutations, data not shown). In another approach, we formulated patient subgroups with mutant amplicon sizes within a range of 15 bp resulting in a total of 5 patient subgroups. After the completion of similar statistical comparison as above for the dichotomous subgroups our overall observation was that the *FLT3*-ITD$^{48\text{-}60bp}$ subgroup showed a profoundly different outcome profile compared to other *FLT3*-ITD^{pos} and *FLT3*-ITD^{neg} patients. Thus we decided to perform detailed analyzes with the following subgroups according to *FLT3*-ITD sizes: *FLT3*-ITD$^{<48bp}$ (n=44), *FLT3*-ITD$^{48\text{-}60bp}$; (n=18) and *FLT3*-ITD$^{>60bp}$; (n=19).

Table 4. Pre-treatment, clinical and molecular characteristics according to *FLT3*-ITD mutation size.

Characteristics	***FLT3*-ITD$^{<48bp}$ (n=44, 11.3%)**		***FLT3*-ITD$^{48-60bp}$ (n=18, 4.6%)**		**P**	***FLT3*-ITD$^{>60bp}$ (n=19, 4.9%)**		**P***	**P****
	number/ median	**%/range**	**number/ median**	**%/range**		**number/ median**	**%/range**		
Age *(median, range)*	47.5	(19-83)	49.5	(22-70)	0.471	49	(18-77)	0.680	0.940
Sex (male/ female; %)	15/29	34.1/65.9	10/8	55.6/44.4	0.157	8/11	42.1/57.9	0.579	0.517
Type of AML *(n, %)*									
De novo	35/43	81.4	14/18	77.8	0.736	18/19	94.7	0.253	0.180
MDS-AML	7/43	16.3	3/18	16.7	1	1/19	5.3	0.416	0.340
t-AML	1/43	2.3	1/18	5.5	0.507	0/19	0.0	1	0.487
FAB *(n, %)*									
M0	1/38	2.6	0/17	0.0	1	1/18	5.6	0.544	1
M1	4/38	10.5	2/17	11.8	1	3/18	16.6	0.669	1
M2	3/38	7.9	4/17	23.5	0.185	1/18	5.6	1	0.177
M3	3/38	7.9	1/17	5.9	1	1/18	5.6	1	1
M4	16/38	42.1	6/17	35.3	0.769	3/18	16.6	0.076	0.264
M5	11/38	28.9	4/17	23.5	0.754	9/18	50.0	0.146	0.164
M6	0/38	0.0	0/17	0.0	na	0/18	0.0	na	na
M7	0/38	0.0	0/17	0.0	na	0/18	0.0	na	na
Laboratory data, *median (range)*									
WBC at diagnosis T/L	60	(2.6-301)	74.2	(.3-334)	0.761	50	(.8-298.1)	0.494	0.355
PLT at diagnosis G/L	44	(12-263)	44	(18-442)	0.436	47	(5-199)	0.929	0.820
Cytogenetics *(n, %)*									
Favourable	4/40	10.0	0/17	0.0	0.306	2/18	11.1	1	0.486
Intermediate	34/40	85.0	16/17	94.1	0.622	14/18	77.8	0.483	0.338
Normal	26/40	65.0	12/17	70.6	0.766	10/18	55.6	0.565	0.489
Adverse	2/40	5.0	1/17	5.9	1	2/18	11.1	0.581	1
Mutations *(n, %)*									
NPM1^{pos}	27/43	62.8	27/43	62.8	1.18	11/18	61.1	1	1

Significant p values are shown in bold. P values present comparisons between *FLT3*-ITD insertions between 48 and 60bp long (48-60bp) and *FLT3*-ITD insertions shorter than 48bp (<48bp), p* values present comparisons between *FLT3*-ITD (<48bp) and *FLT3*-ITD insertions longer than 60bp (>60bp), p** valuse present comparison between *FLT3*-ITD (>60bp) and (48-60bp).

Abbreviations: AML: acute myeloid leukemia; FAB: morphology according to French-American British classification; *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication; *FLT3*-ITD$^{<48bp}$: *FLT3*-ITD with insertions shorter than 48bp; *FLT3*-ITD$^{48-60bp}$: *FLT3*-ITD with insertions between 48bp and 60bp long; *FLT3*-ITD$^{>60bp}$: *FLT3*-ITD with insertions longer than 60bp; MDS-AML: AML evolving from myelodysplastic syndrome; *NPM1* ^{pos}: nucleophosmin 1 positive; PLT: platelet count at diagnosis; t-AML: therapy-related AML; WBC: white blood cell count at diagnosis.

Regarding the clinical and laboratory characteristics, no significant differences were observed between these three groups regarding clinical data (sex, etiology of AML, FAB, laboratory data, cytogenetics and other mutations, see Table 4). *FLT3*-ITD size and load did not correlate with each other (Table 5).

Table 5. Correlation between *FLT3*-ITD mutation load and size.

Characteristics	***FLT3*-ITD$^{<48bp}$ (n=33)**		***FLT3*-ITD$^{48-60bp}$ (n=13)**		***FLT3*-ITD$^{>60bp}$ (n=12)**		**P**
	number	%	number	%	number	%	
***FLT3*-ITD$^{<50\%}$**	26	78.8	7	53.8	10	83.3	0.158
***FLT3*-ITD$^{>50\%}$**	7	21.2	6	46.2	2	16.7	

Abbreviations: *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication; *FLT3*-ITD$^{<48bp}$: *FLT3*-ITD with insertions shorter than 48bp; *FLT3*-ITD$^{48-60bp}$: *FLT3*-ITD with insertions between 48bp and 60bp long; *FLT3*-ITD$^{>60bp}$: *FLT3*-ITD with insertions longer than 60bp; *FLT3*-ITD$^{<50\%}$: *FLT3*-ITD with the insertions load higher than 50%; *FLT3*-ITD$^{>50\%}$: *FLT3*-ITD with the insertions lower than 50%.

4.1.3 Treatment outcome according to *FLT3*-ITD mutation status

4.1.3.1 Presence of *FLT3*-ITD

Treatment outcome was evaluated in 324 patients younger than 60 years old and treated with curative intention in the entire AML cohort including 69 *FLT3*-ITD^{pos} and 255 *FLT3*-ITD^{neg} patients (Table 6). The median follow-up was 59 months (range: 23-131 months). In the entire patient cohort, *FLT3*-ITD^{pos} itself did not associate with adverse prognosis. The probability of survival at 24 months was 40.4%±3.1% for *FLT3*-ITD^{neg} and 31.8%±5.6% for *FLT3*-ITD^{pos} patients. Only relapse rate seemed to be increased (*FLT3*-ITD^{pos} 65% vs. *FLT3*-ITD^{neg} 51%; p=0.081). *FLT3*-ITD alone did not influence survival either in the subgroup with intermediate risk karyotype (n=183) or with normal karyotype (n=134).

Interestingly, there was no association between *FLT3*-ITD mutation and adverse outcome in our cohort (Table 6). This could be explained as an effect of different treatment modalities applied in *FLT3*-ITD^{pos} and in *FLT3*-ITD^{neg} groups. Standard cytarabine and daunorubicin based chemotherapy ("7+3") was administered as induction in 86.9% (n=60/69) of *FLT3*-ITD^{pos} and in 77.6% (n=198/255) of *FLT3*-ITD^{neg} cases (p=0.095), high dose cytarabine as maintenance in 78.8% (n=41/52) of *FLT3*-ITD^{pos} and in 77.4% (n=127/164) of *FLT3*-ITD^{neg} cases (p=1.00). Allogeneic or autologous hematopoietic stem cell transplantation (HSCT, n=49+22 respectively) was performed in 31.9% (n=14+8, 22/69) of *FLT3*-ITD^{pos} and in 19.3% (n=40+9, 49/254) of *FLT3*-ITD^{neg} cases (p=0.033).

Table 6. Treatment outcome according to *FLT3*-ITD mutation status.

Characteristics	*FLT3*-ITD^{neg} (n=255, 78.7%)		*FLT3*-ITD^{pos} (n=69, 21.3%)		p
	number	%	number	%	
Complete remission	174/255	68.2	51/69	73.9	0.382
Early death	37/255	14.5	5/69	7.2	0.156
Resistant disease	44/255	17.2	13/69	18.8	0.725
Relapse	88/174	50.6	33/51	64.7	0.081
Alive	77/255	30.2	19/69	27.5	0.767

Abbreviations: *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication.

As *NPM1* and *FLT3*-ITD mutations frequently associate and influence prognosis in an opposite way, we analyzed the combined effect of *NPM1* and *FLT3*-ITD on survival. Mutant *NPM1* without *FLT3*-ITD (*NPM1*^{pos}- *FLT3*-ITD^{neg}: low risk group) proved to be associated with longer DFS and OS compared to other combinations (*NPM1*^{neg}- *FLT3*-ITD^{neg}, *NPM1*^{pos}- *FLT3*-ITD^{pos}, *NPM1*^{neg}- *FLT3*-ITD^{pos} combined: high risk group) in the intermediate (DFS: p=0.017; OS: p=0.060) and in the normal karyotype group (DFS: p=0.031; OS: p=0.087), but not in the total AML cohort (DFS: p=0.133; OS: p=0.305).

In multivariate analyses, *NPM1*^{pos}- *FLT3*-ITD^{neg} status was associated with longer OS independently of age, cytogenetic risk, and HSCT (HR (95%CI): 0.471 (0.240-0.925); p=0.029), as compared to other *FLT3-NPM1* combinations (data not shown). In addition, similar analyzes in patient subgroups according to cytogenetic alterations (namely intermediate and normal karyotype) indicated similar results however *NPM1*^{pos}- *FLT3*-ITD^{neg} status was significantly associated not only with longer OS, but also longer DFS (data not shown).

4.1.3.2 Treatment outcome according to the *FLT3*-ITD load

Treatment outcome was investigated in 43 *FLT3*-ITD$^{<50\%}$ and 15 *FLT3*-ITD$^{>50\%}$ patients. Complete remission rate was not different in *FLT3*-ITD^{neg} (68.2 %), *FLT3*-ITD$^{<50\%}$ (72.1 %) and *FLT3*-ITD$^{>50\%}$ (60.0 %, p=0.6 vs. *FLT3*-ITD^{neg}*;* p=0.5 vs. *FLT3*-ITD$^{<50\%}$). Early death occurred in 14.5 % of *FLT3*-ITD^{neg}, in 7.0 % of *FLT3*-ITD$^{<50\%}$ and 13.3 % of *FLT3*-ITD$^{>50\%}$ patients (p=1 vs. *FLT3*-ITD^{neg}*;* p=0.6 vs. *FLT3*-ITD$^{<50\%}$). Resistant disease occurred in 17.2 % of *FLT3*-ITD^{neg}, 20.9 % of *FLT3*-ITD$^{<50\%}$ and 26.7 %*FLT3*-ITD$^{>50\%}$ (p=0.3 vs. *FLT3*-ITD^{neg}*;* p=0.7 vs. *FLT3*-ITD$^{<50\%}$). Relapse rate showed an increasing tendency in line with the increasing ITD load, although did not reached the level of significance probably due to the low number of patients (50.6% in *FLT3*-ITD^{neg}, 54.8 % in *FLT3*-ITD$^{<50\%}$ and 77.8% *FLT3*-ITD$^{>50\%}$; p=0.2 vs. *FLT3*-ITD^{neg}*;* p=0.3 vs. *FLT3*-ITD$^{<50\%}$). Regarding the other outcome

parameters, presence of higher *FLT3*-ITD load (>50%) resulted in worse DFS and OS and (p=0.010, 0.038 respectively; Fig. 5).

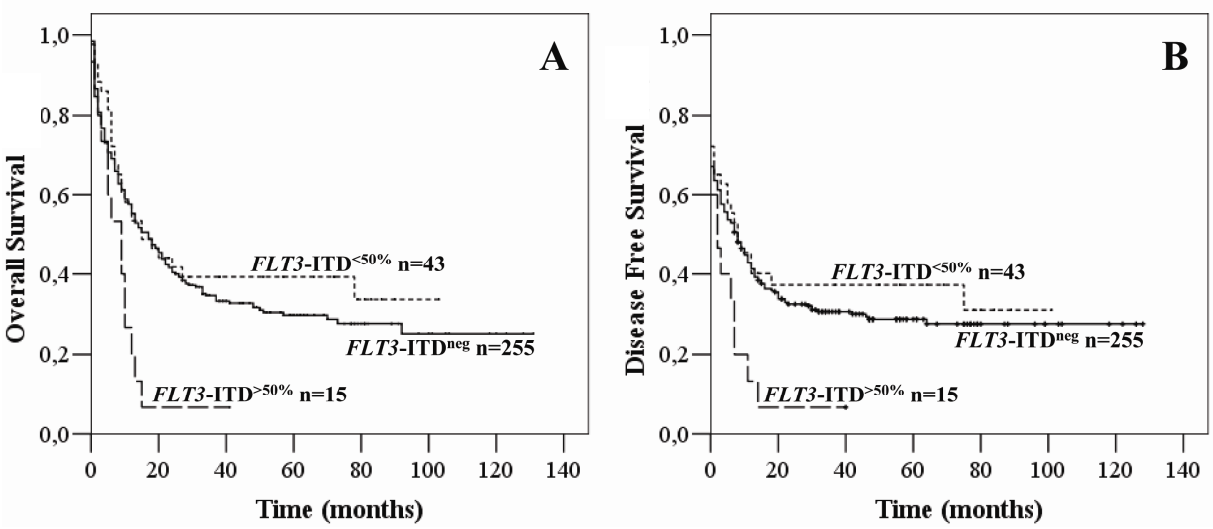

Figure 5. Kaplan-Meier survival analysis of AML patients according to the FLT3-ITD mutation load. (A). Overall survival analysis of AML patients according to the FLT3-ITD mutation load. FLT3-ITD$^{<50\%}$ vs. FLT3-ITD$^{>50\%}$ vs. FLT3-ITD^{neg} p=0.010. (B). Disease free survival analysis of AML patients according to the FLT3-ITD mutation load. FLT3-ITD$^{<50\%}$ vs. FLT3-ITD$^{>50\%}$ vs. FLT3-ITD^{neg} p=0.038.

In the intermediate cytogenetic subgroup, the probability of survival at 24 months was 0.0%±0.0% in *FLT3*-ITD$^{>50\%}$ compared to 41.0%±8.5% in *FLT3*-ITD$^{<50\%}$ (p=0.012), and to 40.8%±4.4% in *FLT3*-ITD^{neg} (p=0.002) subgroups. In the normal karyotype cytogenetic subgroup the probability of survival at 24 months was 0.0%±0.0% in *FLT3*-ITD$^{>50\%}$ compared to 47.7%±1.0% in *FLT3*-ITD$^{<50\%}$ (p=0.012), and to 41.3%±5.1% in *FLT3*-ITD^{neg} (p=0.004) subgroups. In multivariate analyses, *FLT3*-ITD$^{>50\%}$ status was associated with shorter DFS and OS independently of age, cytogenetic risk, and HSCT [HR (95%CI): 2.27 (1.28-4.04); p=0.005 and 2.89 (1.62-5.17); p<0.001], as compared to *FLT3*-ITD^{neg} AML cases (data not shown). In addition, similar analyzes in patient subgroups according to cytogenetics (namely intermediate and normal karyotype) indicated unfavorable effect of *FLT3*-ITD$^{>50\%}$ (data not shown).

4.1.3.3 Treatment outcome according to the size of *FLT3*-ITD

In our first analyses, treatment outcome parameters were compared in two subgroups dichotomized according to ITD size (shorter or longer than 48 bp). Remission rate was not different in patients with *FLT3*-ITD$^{>48bp}$ (n=30, 63.3%) compared to *FLT3*-ITD$^{<48bp}$ (n=38, 81.6%) and *FLT3*-ITD^{neg} (68.2%). Early death rate was higher among *FLT3*-ITD$^{>48bp}$ (16.7%) compared to *FLT3*-ITD$^{<48bp}$ (0.0%, p=0.014). In addition, relapse rate was higher among *FLT3*-ITD$^{>48bp}$ (84.2%) compared to *FLT3*-ITD$^{<48bp}$ (54.8%, p=0.063) or compared to *FLT3*-

ITD^{neg} (50.6%, p=0.007), indicating a significant trend comparing these groups (p=0.011). *FLT3*-ITD$^{>48bp}$ patients displayed shorter DFS and OS compared to *FLT3*-ITD$^{<48bp}$ (p=0.005 and 0.021) or compared to *FLT3*-ITD^{neg} (p=0.013 and 0.040).

As a next step, we compared treatment outcome in the following three subgroups (*FLT3*-ITD$^{<48bp}$, *FLT3*-ITD$^{48\text{-}60bp}$, *FLT3*-ITD$^{>60bp}$). *FLT3*-ITD$^{48\text{-}60bp}$ mutation positive patients showed tendencies towards worse treatment outcome such as lower remission (53.3% vs. 81.6% and 73.3%), higher early death rate (20.0% vs. 0.0% and 13.3%) and relapse rates (87.5% vs. 54.8% and 60.0%) compared to either *FLT3*-ITD$^{<48bp}$ or *FLT3*-ITD$^{>60bp}$ (Table 7).

Table 7. Treatment outcome according to *FLT3*-ITD mutation size.

Characteristics	*FLT3*-ITD$^{<48bp}$ (n=44, 11.3%)		*FLT3*-ITD$^{48-60bp}$ (n=18, 4.6%)		P	*FLT3*-ITD$^{>60bp}$ (n=19, 4.9%)		P*	P**
	number	%	number	%		number	%		
Complete remission	31/38	81.6	8/15	53.3	**0.046**	11/15	73.3	0.708	0.450
Early death	0/38	0.0	3/15	20.0	**0.019**	2/15	13.3	0.076	1
Resistant disease	7/38	18.4	4/15	26.7	0.708	2/15	13.3	1	0.651
Relapse	17/31	54.8	7/8	87.5	0.121	9/15	60.0	1	0.345
Alive	15/38	39.5	0/15	0.0	**0.005**	4/15	26.7	0.528	0.100

Significant p values are shown in bold. P values present comparisons between *FLT3*-ITD insertions between 48 and 60bp long (48-60bp) and *FLT3*-ITD insertions shorter than 48bp (<48bp), p* values present comparisons between *FLT3*-ITD (<48bp) and *FLT3*-ITD insertions longer than 60bp (>60bp), p** values present comparison between *FLT3*-ITD (>60bp) and (48-60bp).

Abbreviations: *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication; *FLT3*-ITD$^{<48bp}$: *FLT3*-ITD with insertions shorter than 48bp; *FLT3*-ITD$^{48-60bp}$: *FLT3*-ITD with insertions between 48bp and 60bp long; *FLT3*-ITD$^{>60bp}$: *FLT3*-ITD with insertions longer than 60bp.

Although none of these differences were significant, collectively these adverse outcome parameters were reflected in a significantly worse DFS and OS (p=0.003, 0.002, Fig. 6).

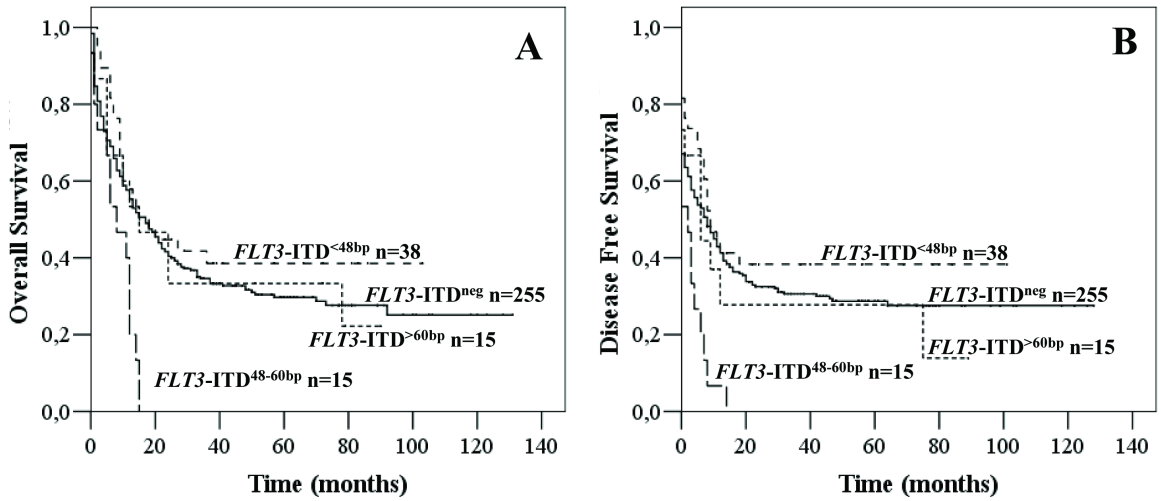

Figure 6. Kaplan-Meier survival analysis of AML patients according to the FLT3-ITD mutation size.
A) Overall survival analysis of AML patients according to the FLT3-ITD mutation size. FLT3-ITD$^{<48bp}$ FLT3-ITD$^{48-60bp}$, FLT3-ITD$^{>48bp}$ vs. FLT3-ITD^{neg} p=0.003.
B) Disease free survival analysis of AML patients according to the FLT3-ITD mutation size. FLT3-ITD$^{<48bp}$, FLT3-ITD$^{48-60bp}$, FLT3-ITD$^{>48bp}$ vs. FLT3-ITD^{neg} p=0.002.

Probability of survival at 24 months was 0.0±0.0% in *FLT3*-ITD$^{48-60bp}$ compared to 40.4±3.1% in *FLT3*-ITD^{neg} (p<0.0001), to 44.7±8.1% in *FLT3*-ITD$^{<48bp}$ (p<0.0001) and to 33.3±12.2% in *FLT3*-ITD$^{>60bp}$ (p=0.014) subgroups. In multivariate analyses (Table 8), *FLT3*-ITD$^{48-60bp}$ was associated with shorter DFS and OS independently of age, cytogenetic risk, and HSCT [HR (95%CI) for DFS: 2.61 (1.52-4.47) and for OS: 2.77 (1.61-4.75)], as compared to *FLT3*-ITD^{neg} AML cases.

Table 8. Multivariate analysis for overall and disease-free survival in the entire AML cohort.

Characteristics	DFS			OS		
	HR	95%CI	p	HR	95%CI	P
Age	1.01	0.99-1.02	0.081	1.01	0.99-1.02	0.134
Karyotype	2.23	1.79-2.79	**0.000**	2.28	1.83-2.85	**0.000**
HSCT	0.40	0.27-0.59	**0.000**	0.37	0.25-0.55	**0.000**
FLT3-ITD$^{<48bp}$	1.00	0.64-1.58	0.989	1.04	0.66-1.63	0.867
FLT3-ITD$^{60-48bp}$	2.61	1.52-4.47	**0.000**	2.77	1.61-4.75	**0.000**
FLT3-ITD$^{>60bp}$	1.09	0.57-2.06	0.804	1.00	0.53-1.90	0.996

Significant p values are shown in bold.
Abbreviations: AML: acute myeloid leukemia; 95%Cl: 95% confidence interval; DFS: disease free survival; *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication; *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication; *FLT3*-ITD$^{<48bp}$: *FLT3*-ITD with insertions shorter than 48bp; *FLT3*-ITD$^{48-60bp}$: *FLT3*-ITD with insertions between 48bp and 60bp long; *FLT3*-ITD$^{>60bp}$: *FLT3*-ITD with insertions longer than 60bp; HR: hazard ratio; HSCT: hematopoietic stem cell transplantation; OS: overall survival.

In similar analyzes in patient subgroups according to cytogenetics (namely intermediate and normal karyotype) indicated the same unfavorable effect of *FLT3*-ITD$^{48\text{-}60bp}$.

4.2 IDH1 and IDH2 mutations in AML

4.2.1 Determination of *IDH1/2* mutations

To characterize the frequencies of *IDH1* and *IDH2* mutations, we screened 376 AML patients by high resolution melting (HRM) (Fig. 7 and 8) and by allele-specific PCR (AS-PCR) (Fig. 9 and 10). HRM for *IDH1/2* was performed in parallel with AS-PCR in patients with normal karyotype (n=149) and in patients with positive AS-PCR screen (n=56). During a continuous melting of the PCR product from 40°C to 95°C with the saturating double stranded DNA dye, the decline of fluorescence was continuously monitored. Amplicons containing any kind of sequence variations (even a point mutation) were distinguished from the wild-type genotype by an altered amplicon melting curve shape (Fig. 7 and 8). The shape of the curve differs not only from the type of the mutation, but also the relative amount of the mutant compared to the wild type in the sample.

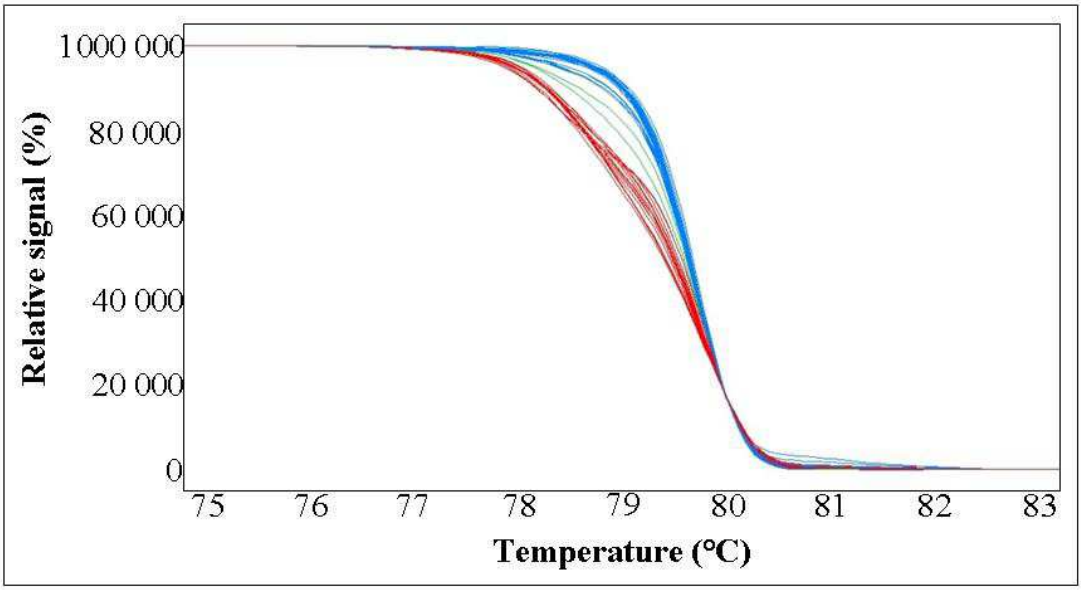

Figure 7.HRM for IDH1.
Normalized and shifted melting curves. Positive samples are presented with red and green color, negative samples with blue color.

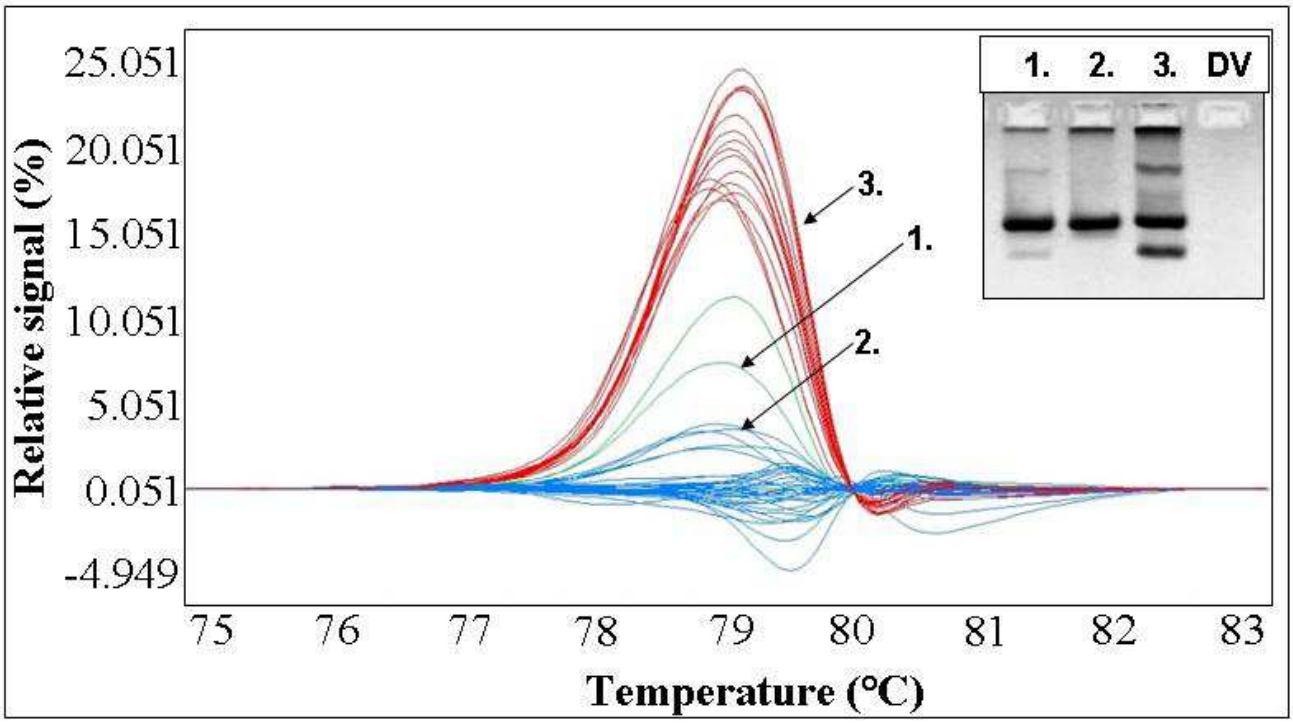

Figure 8.HRM for IDH1.
Normalized and temperature-shifted difference plot. Positive samples are presented with red and green color, negative samples with blue color. The same positive and negative samples (marked with the same number) are presented on agarose gel and HRM difference plot.

To screen *IDH1* gene R132 codon mutations by AS-PCR seven primers were used, including; 2 control primers (Fcont and R), and 5 mutation specific primers (Fspec) (Fig. 9). AS-PCR was followed by gel electrophoresis. In case of *IDH1* negative samples, only 396 bp long band was observed. In case of *IDH1* positive samples two bands were observed: the 396 bp long control band and the 267 bp long mutation specific band. To detect R140Q and R172K mutations four primers were used; including two controls, an *IDH2*-R140Q specific primer, and an *IDH2*-R172K specific primer (Fig. 10). Similarly to *IDH1* AS-PCR was followed by gel electrophoresis. In case of *IDH2* negative samples, only the 399 bp long control band was observed. In case of *IDH2* positive samples two bands were observed, the 399 bp control band and the second one depending on the codon affected by the mutation: 314 bp long for R140Q or 212 bp long for R172K.

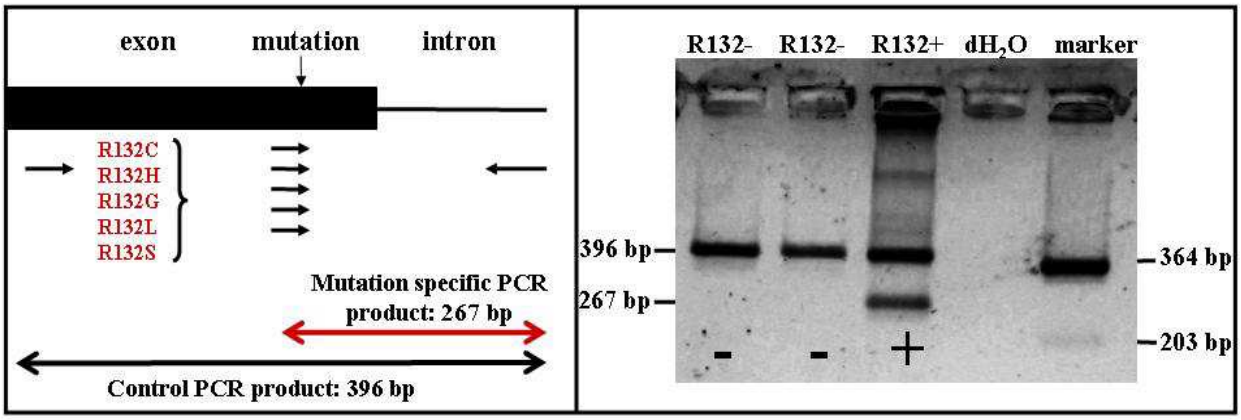

Figure 9. IDH1 allele specific PCR (AS-PCR).
The left panel of the figure shows the location of the primers in the gene, including 5 different mutation specific and 2 control primers. The right panel of the figure illustrates IDH1 AS-PCR products identified using agarose electrophoresis.
Abbreviations: dH_2O: distilled water; R132-: lack of mutation in codon R132 of *IDH1* gene; R132+: presence of mutation in codon R132 of *IDH1* gene.

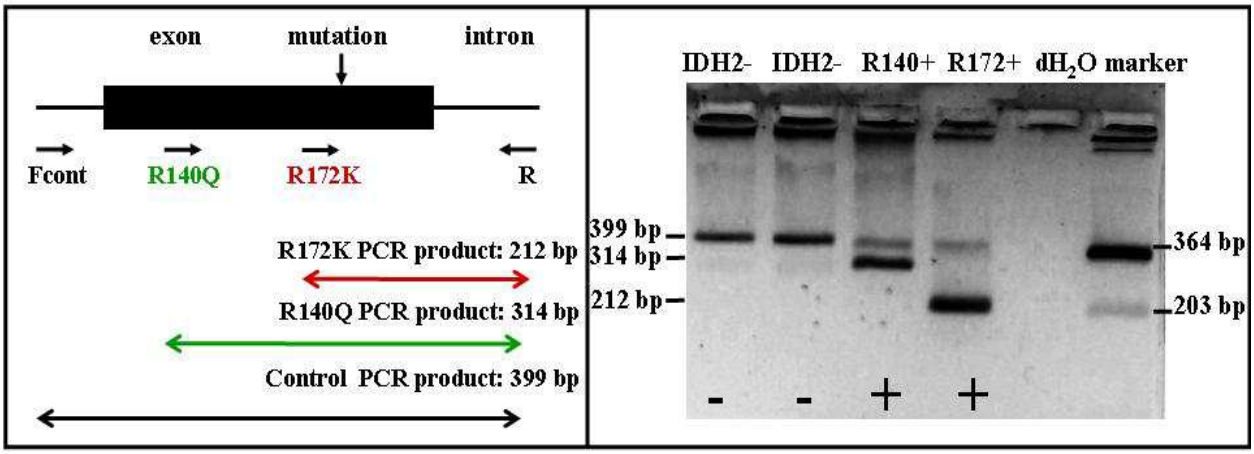

Figure 10. IDH2 allele specific PCR (AS-PCR).
The left panel of the figure shows the location of the primers in the gene, including 2 mutations specific and 2 control primers. Right panel of the figure illustrates IDH2 AS-PCR products identified using agarose electrophoresis.
Abbreviations: dH_2O: distilled water; *IDH2*-: lack of mutation in *IDH2* gene; R140+: presence of mutation in codon R140 of *IDH2* gene; R172+: presence of mutation in codon R172 of *IDH2* gene.

To identify the exact mutation in *IDH1,* we sequenced all *IDH1* samples, which showed positivity by AS-PCR and HRM (Fig. 11). For *IDH2* those samples were sequenced which gave a contradictory results by AS-PCR and HRM (Fig. 12).

Table 9. List of mutations occurring in *IDH1* and *IDH2* genes.

Amino acid change	Nucleotide change	Codon change
IDH1 R132C	c.394C>T	CGT>TGT
IDH1 R132H	c.394G>A	CGT>CAT
IDH1 R132G	c.394C>G	CGT>GGT
IDH1 R132L	c.394G>T	CGT>CTT
IDH1 R132S	c.394C>A	CGT>AGT
IDH2 R140Q	c.419G>A	CGG>CAG
IDH2 R172K	c.515G>A	AGG>AAG

Abbreviations: *IDH1*: Isocitrate dehydrogenase 1*; IDH2:* isocitrate dehydrogenase 2.

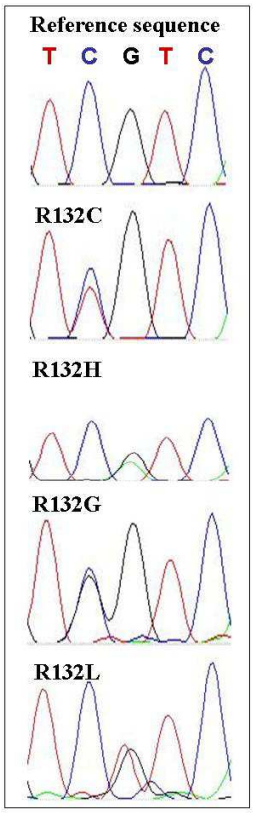

Figure 11. Sequencing of IDH1.
Upper panel shows the reference sequence of IDH1 codon R132 (wild type). Lower panels illustrate IDH1 R132C, R132H, R132G and R132L mutations respectively.

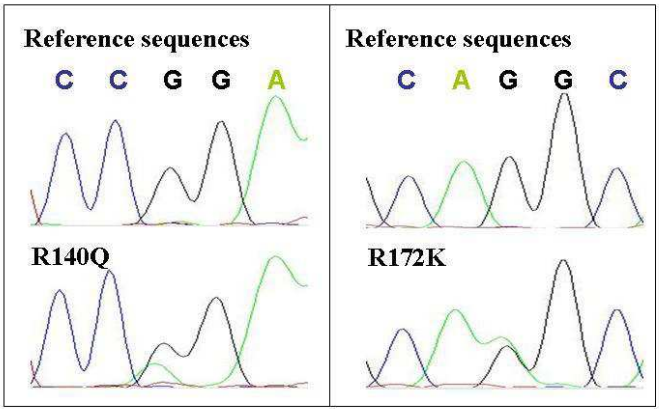

Figure 12. Sequencing of IDH2.
Upper panel shows the reference sequence of IDH2 R140 and R172 codons respectively (wild type). Lower panels illustrate IDH2 R140Q, and R172K mutations respectively.

Altogether 32 patients had a mutation in *IDH1* (8.5%) and 28 patients in *IDH2* (7.5%). *IDH1* and *IDH2* mutations were mutually exclusive, as they never occurred together in the same patient. In *IDH1,* R132C (n=14, 43.8%) was the most frequent alteration. In addition, R132H (n=10, 31.3%), R132G (n=5, 15.6%), R132L (n=2, 6.2%) and R132S (n=1, 3.1%) were detected. In the *IDH2* gene, twenty R140Q (71.4%) and eight R172K (28.6%) substitutions were identified.

4.2.2 Clinical features of *IDH* positive AML

First, we investigated *IDH1* and *IDH2* mutation positive patients combined (*IDH1/2*^{pos}), which was followed by the analysis of *IDH1*^{pos} and *IDH2*^{pos} subtypes separately (Table 10). Then a further stratification was performed for the separate analysis comparing the *IDH1* R132C with the R132H and the *IDH2* R140Q with the R172K mutations (Table 11). We compared the *IDH* mutation positive to the *IDH1/2* double negative patients (*IDH1/2*^{neg}).

Table 10. Pre-treatment, clinical and molecular characteristics according to *IDH1* and *IDH2* mutation status.

Characteristics in the entire AML cohort	***IDH 1/2*** ^{neg} **n=316 (84.1%)**		***IDH1*** ^{pos} **n=32 (8.5%)**		***P***	***IDH2*** ^{pos} **n=28 (7.4%)**		***P***
	Number/ median	**%/ range**	**Number/ median**	**%/ range**		**Number/ median**	**%/ range**	
Age *median, (range)* (n=376)	49.0	(16-86)	54.5	(30-93)	**0.013**	56.5	(31-77)	**0.009**
Sex *(male/ female; %)*	156/160	(49.4%/50.6%)	11/21	(34.4/65.6%)	0.137	13/15	(46.4/53.6%)	0.845
Type of AML *(n, %)*								
De novo	190/314	(60.5%)	22/32	(68.8%)	0.447	20/26	(76.9%)	0.140
MDS-AML	101/314	(32.2%)	6/32	(18.8%)	0.159	6/26	(23.1%)	0.388
t-AML	23/314	(7.3%)	4/32	(12.4%)	0.296	0/26	(0.0%)	0.238
FAB *(n, %)*								
M0	11/282	(3.9%)	0/29	(0.0%)	0.608	0/25	(0.0%)	0.609
M1	66/282	(23.4%)	8/29	(27.6%)	0.648	10/25	(40.0%)	0.088
M2	37/282	(13.1%)	6/29	(20.7%)	0.261	0/25	(0.0%)	0.055
M3	31/282	(10.9%)	0/29	(0.0%)	0.094	2/25	(8.0%)	1
M4	78/282	(27.7%)	11/29	(37.9%)	0.281	9/25	(36.0%)	0.364
M5	53/282	(18.8%)	4/29	(13.8%)	0.621	4/25	(16.0%)	1
M6	3/282	(1.1%)	0/29	(0.0%)	1	0/25	(0.0%)	1
M7	3/282	(1.1%)	0/29	(0.0%)	1	0/25	(0.0%)	1
Laboratory data, *median (range)*								
WBC, T/L (n=347)	10.5	(0.3-368)	10.7	(0.09-301)	0.832	6.91	(0.8-300)	0.294
PLT, G/L (n=278)	39	(5-684)	75	(10-326)	**0.039**	72	(5-215)	**0.005**
Cytogenetics *(n, %)*								
Favourable	61/300	(20.3%)	2/31	(6.5%)	0.089	2/27	(7.4%)	0.128
Intermediate	158/300	(52.7%)	25/31	(80.6%)	**0.004**	22/27	(81.5%)	**0.004**
Adverse	81/300	(27.0%)	4/31	(12.9%)	0.128	3/27	(11.1%)	0.105
Mutations *(n, %)*								
FLT3-ITD^{pos}	70/315	(22.2%)	6/32	(18.8%)	0.823	4/28	(14.3%)	0.472
NPM1^{pos}	73/315	(23.2%)	13/31	(42.0%)	**0.029**	9/28	(33.3%)	0.244

Significant p values are shown in bold.

Abbreviations: AML: acute myeloid leukemia; FAB: morphology according to French-American British classification; *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication; *IDH*: isocitrate dehydrogenase; MDS-AML: AML evolving from myelodysplastic syndrome; *NPM1*: nucleophosmin 1; PLT: platelet count at diagnosis; t-AML: therapy-related AML; WBC: white blood cell count at diagnosis.

Table 11. Pre-treatment, clinical and molecular characteristics of *IDH1* R132C, R132H and *IDH2* R140Q, R172K mutations.

Characteristics	**R132C+ n=14 (3.7%)**		***P***	**R132H+ n=10 (2.7%)**		***P***	***P****	**R140Q+ n=20 (5.3%)**		***P***	**R172K+ n=8 (2.1%)**		***P***	***P#***
	Number/ median	**%/ range**		**Number/ median**	**%/ range**			**Number/ median**	**%/ range**		**Number/ median**	**%/ range**		
Age *median,(range)*	57	(33-93)	**0.024**	52	(30-66)	0.561	0.285	56.5	(40-77)	**0.009**	56.5	(31-66)	0.415	0.746
Sex *(male/female; %)*	5/9	(35.7/64.3%)	0.416	3/7	(30.0/70.0.%)	0.338	0.56	11/9	(55.0/45.0%)	0.652	2/6	(25.0/75.0%)	0.284	0.16
Type of AML*(n, %)*														
De novo	7/14	(50.0%)	0.578	9/10	(90.0%)	0.095	0.08	15/18	(83.3%)	0.078	5/8	(62.5%)	1	0.33
MDS-AML	4/14	(28.6%)	1	1/10	(10.0%)	0.179	0.36	3/18	(16.7%)	0.201	3/8	(37.5%)	0.716	1
t-AML	3/14	(21.4%)	0.089	0/10	(0.0%)	1	0.24	0/18	(0.0%)	0.624	0/8	(0.0%)	1	1
FAB *(n, %)*														
M0	0/12	(0.0%)	1	0/9	(0.0%)	1	-	0/20	(0.0%)	1	0/5	(0.0%)	1	-
M1	6/12	(50.0%)	0.078	0/9	(0.0%)	0.217	**0.02**	9/20	(45.0%)	0.056	1/5	(20.0%)	1	0.61
M2	1/12	(8.3%)	1	3/9	(3.3%)	0.112	0.27	0/20	(0.0%)	0.149	0/5	(0.0%)	1	-
M3	0/12	(0.0%)	0.623	0/9	(0.0%)	0.604	-	2/20	(10.0%)	1	0/5	(0.0%)	1	1
M4	4/12	(33.3%)	0.744	4/9	(44.4%)	0.275	0.67	5/20	(25.0%)	1	4/5	(80.0%)	**0.025**	**0.04**
M5	1/12	(8.3%)	0.702	2/9	(22.2%)	0.680	0.55	4/20	(20.0%)	1	0/5	(0.0%)	0.588	0.55
M6	0/12	(0.0%)	1	0/9	(0.0%)	1	-	0/20	(0.0%)	1	0/5	(0.0%)	1	-
M7	0/12	(0.0%)	1	0/9	(0.0%)	1	-	0/20	(0.0%)	1	0/5	(0.0%)	1	-
Laboratory data, *median(range)*														
WBC, T/L	8.4	(1-301)	0.685	14.5	(0.09-100)	0.946	0.794	12.4	(0.8-300)	0.846	22.0	(1.2-25.1)	**0.019**	**0.028**
PLT, G/L	45	(10-154)	0.982	136	(25-326)	**0.017**	**0.050**	68.5	(5-215)	**0.015**	79	(12-140)	0.132	0.929
Cytogenetics *(n, %)*														
Favourable	0/13	(0.0%)	0.080	2/10	(20.0%)	1	0.18	2/19	(10.5%)	0.386	0/8	(0.0%)	0.364	1
Intermediate	9/13	(69.2%)	0.271	8/10	(80.0%)	0.113	0.66	15/19	(79.0%)	0.583	7/8	(87.5%)	0.072	1
Adverse	4/13	(30.8%)	0.755	0/10	(0.0%)	0.069	0.10	2/19	(10.5%)	0.175	1/8	(12.5%)	0.686	1
Mutations *(n, %)*														
FLT3-ITD^{pos}	2/14	(14.3%)	0.742	2/10	(20.0%)	1	0.82	4/20	(20.0%)	1	0/8	(0.0%)	0.209	0.240
NPM1^{pos}	2/14	(14.3%)	0.744	7/10	(70.0%)	**0.003**	**0.02**	9/19	(47.4%)	**0.026**	0/8	(0.0%)	0.206	**0.020**

Significant p values are shown in bold. P values present comparisons between *IDH* mutation positive and *IDH1/2* double negative (*IDH1/2*^{neg}) patients. P* values present comparisons between *IDH1* R132C and R132H, p# values present comparisons between *IDH2* R140Q and R172K mutation positive patients.

Abbreviations: AML: acute myeloid leukemia; FAB: morphology according to French-American British classification; *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication; MDS-AML: AML evolving from myelodysplastic syndrome; *NPM1*: nucleophosmin 1; PLT: platelet count at diagnosis; t-AML: therapy-related AML; WBC: white blood cell count at diagnosis.

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The *IDH1/2* mutations together, as well as the *IDH1* and *IDH2* separately including the R132C and R140Q subtypes presented at older age (medians and ranges are listed) [*IDH1*^{pos}: 54.5 (30-93 years); *IDH2*^{pos}: 56.5 (31-77 years) compared to the *IDH1/2*^{neg}: 49.0 (16-86 years); p=0.013 and p=0.009, respectively]. The *IDH* mutations showed no association with sex, etiology of AML or white blood cell (WBC) count at diagnosis. Higher platelet count (PLT) was observed in the *IDH1/2*^{pos}, *IDH1*^{pos} and *IDH2*^{pos} as well as in the R132C, R132H and R140Q mutants [*IDH1*^{pos}: 75 (10-326 G/L); *IDH2*^{pos}: 72 (5-215 G/L); vs. *IDH1/2*^{neg}: 39 (5-684 G/L); p=0.039, p=0.005]. No difference could be observed in morphological distribution according to FAB.

The *IDH1* and *IDH2* mutation positive patients had intermediate risk karyotype more frequently [*IDH1*^{pos}*:* 80.6%, *IDH2*^{pos}*:* 81.5% vs. *IDH1/2*^{neg}*:* 52.7%; p=0.004, p=0.004]. None of the *IDH* substitutions were preferentially associated with *FLT3*-ITD. The *IDH1/2* and *IDH1* positive patients had predominantly *NPM1* (*IDH1*^{pos}*:* 42.0% vs. *IDH1/2*^{neg}*:* 23.2%; p=0.029), while the *IDH2* was not associated with *NPM1* (33.3%, p=0.24).

Interestingly, marked differences in the clinical presentation were observed between *IDH1* R132H and R132C mutant AML patients. R132H and R132C comparisons are noted with p* in Table 11, while p values reflect comparisons to *IDH1/2*^{neg}. R132H mutant AMLs were more likely to have de novo origin (90%), while R132C positive AMLs were secondary to myelodysplastic syndrome (MDS), or therapy related in 50% of cases (p=0.08). FAB M1 was more common in R132C (50% vs. 0%, p=0.02). PLT count at diagnosis was higher in R132H (136 vs. 45 G/L; p=0.050). The R132H mutated AML was more likely to associate with *NPM1* than R132C (70% vs. 14%; p=0.02). Several distinctive features were also detected between *IDH2* R140Q and R172K mutation carriers at diagnosis (comparisons are noted with p# in Table XIV). R172K showed lower WBC (12.4 T/L vs. 22.0 T/L; p=0.028). R172K mutation was mutually exclusive with *NPM1* (0% vs. R140Q: 47.4%; p=0.02).

In order to confirm our findings in more homogenous cohorts we also analyzed patients with *de novo* AML of consisting of 232 patients. 22 *IDH1* (9.5%) and 20 *IDH2* mutations (8.6%) were identified. Similarly to the total AML cohort, we observed older age at diagnosis in *IDH2*^{pos} (54.0 vs. 47.0 years; p=0.02), higher PLT count in *IDH1*^{pos} (93 vs. 37G/L; p=0.009), and in *IDH2*^{pos}, (66G/L; p=0.035), higher frequency of intermediate risk karyotype in *IDH1*^{pos} (90.5 vs. 65.0%; p=0.024), and in *IDH2*^{pos} (89.5%; p=0.038), higher frequency of *NPM1* mutation in *IDH1*^{pos} (57.1 vs. 32.6%; p=0.03), compared to *IDH1/2*^{neg}.

In the intermediate cytogenetic risk group consisting of 205 patients, 25 *IDH1* (12.2%) and 22 *IDH2* mutations (10.7%) were identified. Similarly to the total AML group, *IDH* mutations

occurred more often at older age in *IDH1*^{pos} (p=0.041), and in *IDH2*^{pos}, (p=0.052) and were associated with higher PLT count at diagnosis in *IDH2*^{pos}, (p=0.029). The R172K was associated with lower WBC count compared not only to the R140Q, but also to the *IDH1/2*^{neg} subgroup. The R140Q positive tumors preferentially showed FAB M1 subtype [R140Q: 53.3% vs. *IDH1/2*^{neg}*:* 24.8%, p=0.03]. The R172K cases were more likely to have an intermediate risk and abnormal karyotype than the R140Q samples (28.6% vs. 80.0 %; p=0.052). *IDH1* R132H is preferentially associated with *NPM1* (87.5%) when compared to *IDH1/2*^{neg} (38.9%; p=0.009) or to R132C cases (11.1%; p=0.003). *IDH2* R172K was mutually exclusive with *NPM1* (0% vs. *IDH1/2*^{neg}: 38.9%, R140Q: 53.3%; p=0.047, 0.022 respectively). *IDH1/2*^{pos} patients were less likely to carry *FLT3*-ITD mutations (*IDH1/2*^{pos}: 19.1% vs. *IDH1/2*^{neg}: 35.0%, p=0.048).

The *IDH1/2*, *IDH2* as well as the R132C and R140Q mutants showed lower mRNA expression of *ABCG2* gene at diagnosis [*IDH2*^{pos}*:* 0.51 (0.05-3.24%) vs. *IDH1/2*^{neg}: 1.35 (0.02-18.05%); p=0.012]. Also, *IDH1*^{pos} showed a tendency toward a lower expression [0.52 (0.01-23.01%); p=0.062]. On the other hand, the *ABCG2* expression was significantly lower in *NPM1* positive as compared to the *NPM1* negative AML samples [*NPM1*^{pos}: 0.035 (0.01-2.02%) vs. *NPM1*^{neg}: 1.028 (0.02-23.01%); p<0.001]. To test whether the association of *IDH* mutations with a lower *ABCG2* expression was independent from *NPM1*, we divided patients into three groups according to their *IDH1/2* and *NPM1* mutational status (Fig. 13).

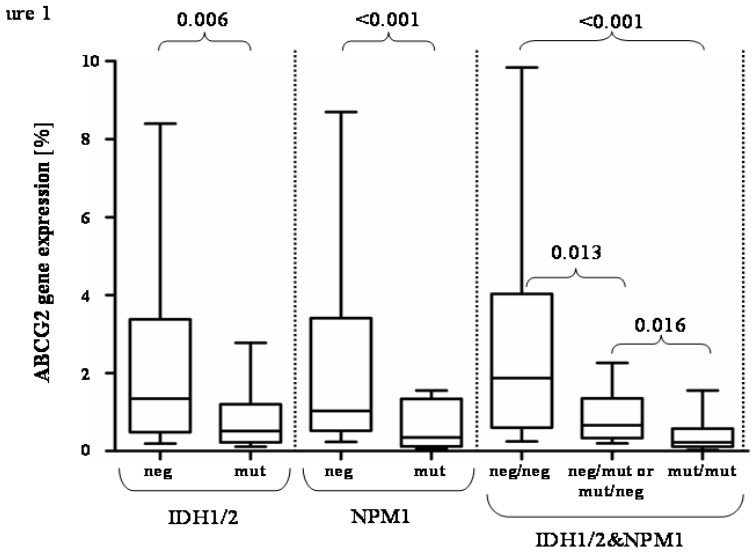

Figure 13. Boxplot expression (median, 25th and 75th quartiles are shown on the boxes, 10th and 90th percentiles are presented as error bars) of ABCG2 expression in AML according to IDH1/2 mutation status alone, to NPM1 mutation status alone and IDH1/2 and NPM1 mutation status combined.

ABCG2 mRNA expression was the highest in the double negative group [*IDH1/2*^{neg}/*NPM1*^{neg}: 1.87 (0.02-18.05%)] comparing to the single positive group [*IDH1/2*^{neg}/*NPM1*^{pos} and *IDH1/2*^{pos}/*NPM1*^{neg}: 0.62 (0.07-23.01%); p=0.013] and to the double positive group [*IDH1/2*^{pos}/*NPM1*^{pos}: 0.22 (0.01-1.61%); p<0.001). In order to confirm our findings in more homogenous cohorts, we also analyzed the subgroup of patients with *de novo* AML consisting of 232 patients. 22 *IDH1* (9.5%) and 20 *IDH2* mutations (8.6%) were identified. Similarly to the total AML group, we noticed a higher *ABCG2* mRNA level in the double *IDH1/2*^{neg}/*NPM1*^{neg} group compared to the *IDH1/2*^{neg}/*NPM1*^{pos} or the *IDH1/2*^{pos}/*NPM1*^{neg} single positive and the *IDH1/2*^{pos}/*NPM1*^{pos} double positive subgroups (3.53 (0.15-18.05%) vs. 0.66 (0.07-2.02%); 0.35 (0.05-1.61%), p=0.002; 0.001 respectively)

4.2.3 Impact of *IDH* mutations on clinical outcome

Clinical outcome was evaluated in 314 patients younger than 60 years old and treated with curative intention in the entire AML group, including 45 *IDH1/2*^{pos} and 269 *IDH1/2*^{neg} patients (Tables 12 and 13).

Table 12. Treatment outcome according to *IDH1* and *IDH2* mutation status.

	IDH 1/2 ^{neg} **n=316 (84.1%)**		***IDH1*** ^{pos} **n=32 (8.5%)**		***P***	***IDH2*** ^{pos} **n=28 (7.4%)**		***P***
	Number	**(%)**	**Number**	**(%)**		**Number**	**(%)**	
Complete remission	184/269	(68.4%)	17/24	(70.8%)	1	18/21	(85.7%)	0.138
Early death	34/269	(12.6%)	5/24	(20.8%)	0.361	1/21	(4.8%)	0.487
Resistant disease	51/269	(19.0%)	2/24	(8.3%)	0.272	2/21	(9.5%)	0.387
Relapse	98/184	(53.3%)	9/17	(52.9%)	1	11/18	(61.1%)	0.624
Alive	81/269	(30.1%)	5/24	(20.8%)	0.483	9/21	(42.9%)	0.229

Abbreviations: *IDH*: isocitrate dehydrogenase.

Table 13. Treatment outcome according to *IDH1* R132C, R132H and *IDH2* R140Q, R172K mutations.

	R132C+ n=14 (3.7%)		***P***	**R132H+ n=10 (2.7%)**		***P***	***P****	**R140Q+ n=20 (5.3%)**		***P***	**R172K+ n=8 (2.1%)**		***P***	***P#***
	Number	**(%)**		**Number**	**(%)**			**Number**	**(%)**		**Number**	**(%)**		
Complete remission	7/9	(77.8%)	0.725	5/9	(55.6%)	0.474	0.619	13/16	(81.3%)	0.406	5/5	(100.0%)	0.329	0.549
Early death	1/9	(11.1%)	1	4/9	(44.4%)	**0.023**	0.294	1/16	(6.3%)	0.702	0/5	(0.0%)	1	1
Resistant disease	1/9	(11.1%)	1	0/9	(0.0%)	0.373	1	2/16	(12.5%)	0.745	0/5	(0.0%)	0.588	1
Relapse	4/7	(57.1%)	1	4/5	(80.0%)	0.376	0.576	8/13	(61.5%)	0.775	3/5	(60.0%)	1	1
Alive	2/9	(22.2%)	1	0/9	(0.0%)	0.063	0.471	6/16	(37.5%)	0.579	3/5	(60.0%)	0.169	0.611

P values present comparisons between *IDH* mutation positive and *IDH1/2* double negative (*IDH1/2*^{neg}) patients. P* values present comparisons between *IDH1* R132C and R132H, p# values present comparisons between *IDH2* R140Q and R172K mutation positive patients.

Abbreviations: *IDH*: isocitrate dehydrogenase.

The *IDH1*^{pos} and the *IDH2*^{pos} patients had similar remission and relapse rates compared to the *IDH1/2*^{neg} patients. OS and DFS were not altered in the *IDH1*^{pos} or the *IDH2*^{pos} AML. On the other hand, a detailed analysis of the prognostic impact of different mutations revealed differences between particular *IDH* substitutions (Fig. 14).

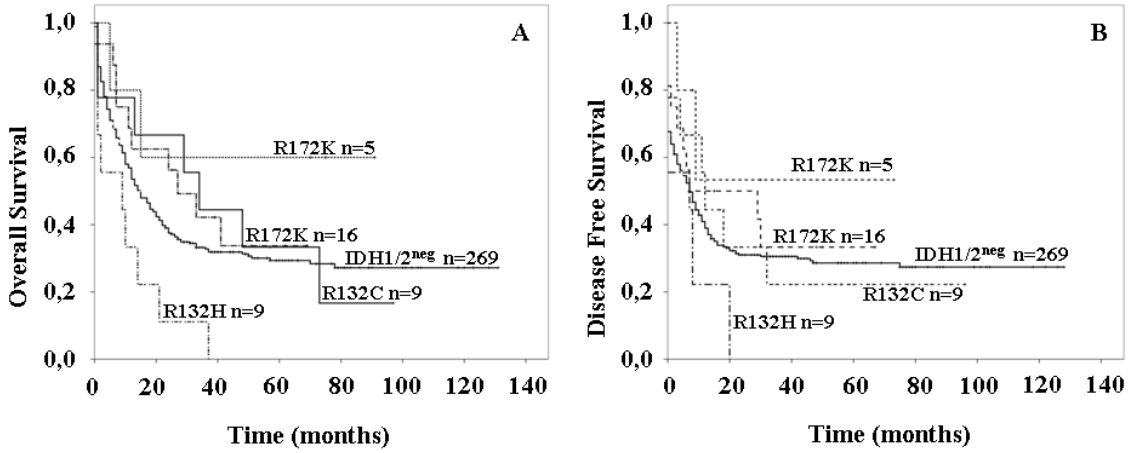

Figure 14. Kaplan-Meier survival analysis of AML patients according to different IDH1 and IDH2 mutations.
(A). Overall survival analysis of AML patients according to the different IDH1 and IDH2 mutations. R132H, R132C, R140Q, R172K vs. IDH1/2^{neg} *p=0.02, 0.742, 0.357, 0.197 respectively; R132C vs. R132H p=0.019; R140Q vs. R172K p=0.455.*
(B). Disease free survival analysis of AML patients according to the different IDH1 and IDH2 mutations. R132H, R132C, R140Q, R172K vs. IDH1/2^{neg} *p=0.091, 0.892, 0.545, 0.253 respectively; R132C vs. R132H p=0.122; R140Q vs. R172K p=0.399*

Patients harboring the *IDH1* R132H had a higher early death rate (R132H: 44.4% vs. *IDH1/2*^{neg}: 12.6%; p=0.023), resulting in shorter OS for the R132H patients compared to the *IDH1/2*^{neg} (p=0.02) or the R132C carriers (p=0.019). The 4-year OS was 0% in R132H, 33% in R132C, and 31% in *IDH1/2*^{neg} AML patients. In multivariate analyses (Table 14), *IDH1* R132H was associated with shorter OS independently of age, WBC count, cytogenetic risk, and *NPM1*-*FLT3*-ITD status [HR (95%CI): 2.92 (1.38-6.16)], as compared to *IDH1/2*^{neg} AML cases.

Table 14. Multivariate analysis for overall and disease-free survival in all AML patients.

	Total AML					
	OS			DFS		
	HR	95%Cl	p	HR	95%Cl	p
Age	1.02	1.00-1.03	**0.002**	1.02	1.00-1.03	**0.002**
Karyotype	2.09	1.68-2.62	**0.000**	2.04	1.64-2.54	**0.000**
NPM1-FLT3-ITD risk*	0.63	0.39-1.04	0.069	0.60	0.37-0.97	**0.041**
R132H mutation**	2.92	1.44-5.89	**0.003**	2.28	1.13-4.58	**0.021**

Significant p values are shown in bold.

Abbreviations: AML: acute myeloid leukemia; 95%Cl: 95% confidence interval; DFS: disease free survival; *FLT3*-ITD: fms-like tyrosine kinase internal tandem duplication; HR: hazard ratio; *NPM1*: nucleophosmin 1; OS: overall survival.

Remarks:* *NPM1-FLT3-ITD* risk: low risk (*NPM1*^{mut} and *FLT-ITD3*^{neg}) vs. high risk group (*NPM1*^{neg} and *FLT3-ITD*^{mut}, *NPM1*^{mut} and *FLT3-ITD*^{mut}, *NPM1*^{neg} and *FLT3-ITD*^{mut} combined). ** R132H positive patients vs. *IDH1/2*^{neg} patients.

Treatment modalities were not different between patients with different *IDH* mutations status. Most patients (*IDH1*^{pos}: 92 %; *IDH2*^{pos}: 95% vs. *IDH1/2*^{neg}:77%) received standard daunorubicin and cytarabine containing therapeutic regimens as induction (p=0.12; 0.06 respectively) and high-dose cytarabine as post-remission therapy (*IDH1*^{pos}: 71%; *IDH2*^{pos}: 89% vs. *IDH1/2*^{neg}: 73%; p=0.78; 0.17 respectively). R132H positive individuals received similar induction (89%; p=0.69) and post-remission regiments (100%; p=0.32); thus therapeutic variability is unlikely to be responsible for the observed differences in outcome. Inclusion of treatment in multivariate analysis did not influence the adverse survival impact of R132H.

In *de novo* AML (n=203), 20 *IDH1*^{pos} and 18 *IDH2*^{pos} patients were received induction chemotherapy. *IDH1*^{pos} and R132H patients had a higher early death rate comparing to *IDH1/2*^{neg} (25.0; 50.0 vs. 9.1%; p=0.047; 0.006) resulting in inferior OS (p=0.086; 0.001) compared to *IDH1/2*^{neg}. In the intermediate cytogenetic risk group, we evaluated 177 patients for clinical outcome, including 38 *IDH1/2*^{pos} and 139 *IDH1/2*^{neg} patients (data not shown). Similarly to the entire AML cohort, there were no significant differences in remission and relapse rates, OS and DFS between the patients with or without the *IDH1/2* mutations. The patients harboring R132H had a higher early death rate (42.9% vs. 9.4%; p=0.029). The R132H also showed a tendency toward adverse OS compared to the *IDH1/2*^{neg} (p=0.09) and to the R132C (p=0.052) groups.

4.3 ABCG2 expression on red blood cell membranes in healthy individuals

4.3.1 Determination of ABCG2 expression by flow cytometry

To quantify ABCG2 expression on the red blood cell (RBC) membrane, flow cytometry based assay was used. Anticoagulated blood samples of healthy volunteers were stained with monoclonal antibodies (mAb) recognizing human ABCG2, and subjected to flow cytometry (Fig. 15). Antibody staining was performed by BXP34, BXP21 and by 5D3 mAbs specific for ABCG2, or the respective IgG control antibodies, followed by staining with PE-labeled secondary antibodies. BXP34 and BXP21 recognize intracellular ABCG2 epitope. After staining with these antibodies permeabilised RBC membranes, called ghost showed high intensity labeling, while non-permeabilised cells had low intensity labeling, roughly equal to that of the IgG control. 5D3 recognizes extracellular ABCG2 epitop and in this case both permeabilised and non-permeabilised cells showed high intensity labeling.

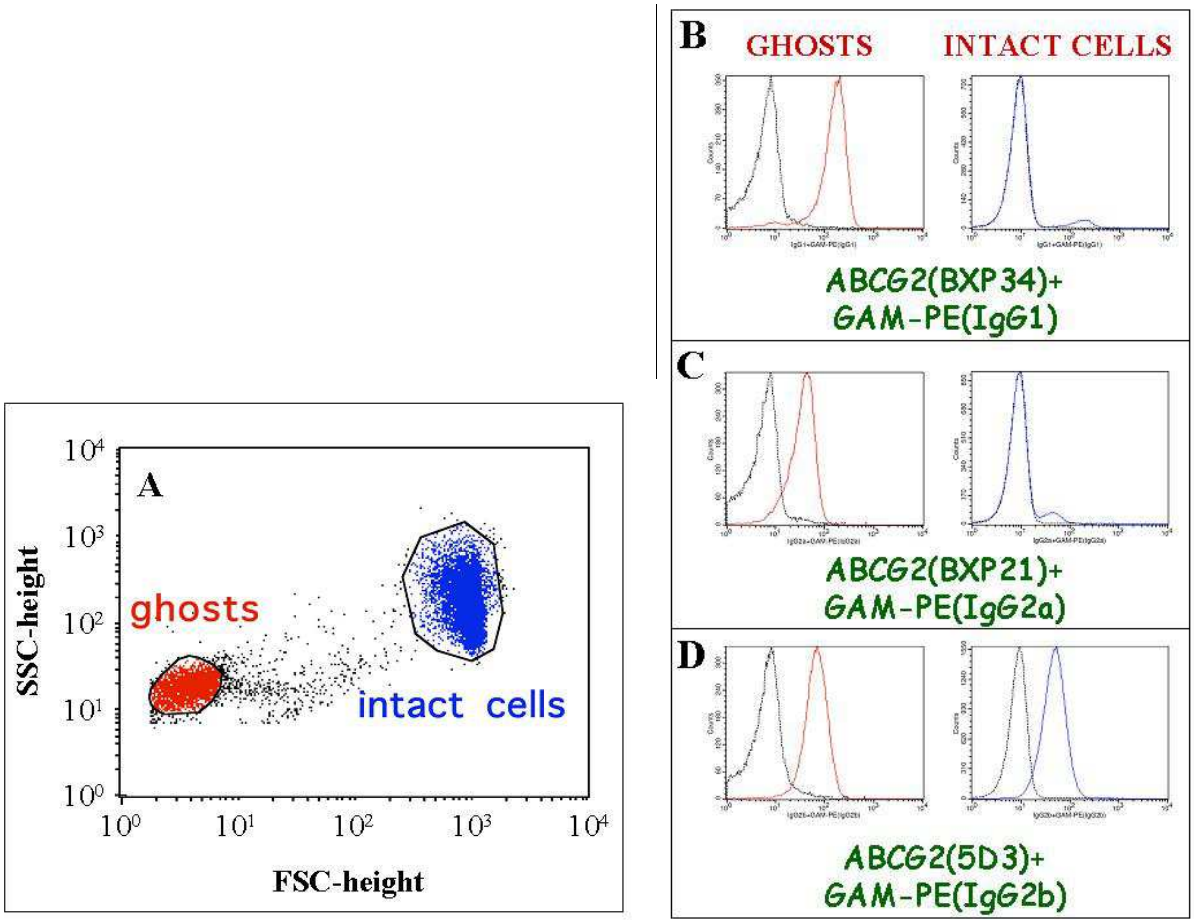

Figure 15. Quantitative determination of ABCG2 expression in the erythrocyte membrane by flow cytometry. A) Intact cells and ghost gated based on the forward scatter (FSC) and side scatter (SSC) parameters. B) Antibody staining was performed by BXP34 (B), BXP21(C) and by 5D3 (D) mAbs specific for ABCG2, or the respective IgG control antibodies, followed by staining with PE-labeled secondary antibodies.

As documented in Fig. 15, all three ABCG2-specific antibodies (BXP34, BXP21, 5D3) detected significant expression of ABCG2 on the red blood cell membrane. All antibodies were titrated in order to allow quantitative protein determination and to obtain maximum binding. After antibody titration all the relevant monoclonal antibodies were applied in concentrations exceeding maximum binding levels. Since the exact epitopes of the ABCG2 mAbs are unknown, and BXP34 (Scheffer; 2000) labeling consistently gave three times greater relative staining than either the BXP21 (Diestra; 2002) or the 5D3 antibodies (Ozvegy-Laczka; 2008), for ABCG2 expression, a weighted average (BXP34/3+BXP21)/2, named "RBC-G2 factor" was used. A linear correlation between the weighted average binding of the two mAbs, BXP34 and BXP21, recognizing intracellular epitopes, and the binding of the cell-surface reactive 5D3 mAb was observed. As a control lysed rat and pig red cells were used to analyze the specificity of the anti-ABCG2 antibodies applied. None of the three human-specific anti-ABCG2 antibodies and the anti-glycophorin A monoclonal antibody labeled rat or pig red cells.

The intra-assay imprecision (coefficient of variation [CV%] expressed as SD/mean %) for the ABCG2 factor was 5.3%, as measured from 8 peripheral blood aliquots from the same healthy volunteer on the same day. Inter-assay imprecision was calculated from samples taken from the same individual on different days. An inter-assay CV% for ABCG2 factor of 10.1% was obtained, when using results received from 29 individuals with replicate measurements (using 2 - 4 replicates in each case).

4.3.2 ABCG2 expression in healthy carriers of *ABCG2* polymorphic variants

Significant ABCG2 levels, encompassing a wide range of expression were detected in the red blood cells of all individuals. Differences of the erythrocyte ABCG2 expression could not be attributed to age or sex. Thereafter, to examine if the red cell membrane ABCG2 protein levels correlated with pharmacologically relevant polymorphisms that are known to influence protein expression in model cells, 47 unrelated, healthy individuals were screened to examine the expression of two most prevalent *ABCG2* polymorphic variants found in the Caucasian population (V12M and Q141K) (Cervenak; 2006). Among the 47 donors 11 individuals with the heterozygous presence of the DNA sequence coding for the Q141K variant (carrier frequency: 23.4%, allele frequency: 11.7±6.6%), and 3 individuals with the heterozygous presence of the V12M variant (carrier frequency: 6.4%; allele frequency: 3.2±3.6%) were

found. The red blood cells of individuals carrying the heterozygous Q141K variant exhibited significantly lower expression of ABCG2 (5.27±1.19), as compared to homozygous wild-type individuals (6.13±0.61, p= 0.011) (Fig. 16). There was no significant difference between homozygous wild-type individuals and heterozygous V12M carriers, although the number of the carriers of this variant was relatively low.

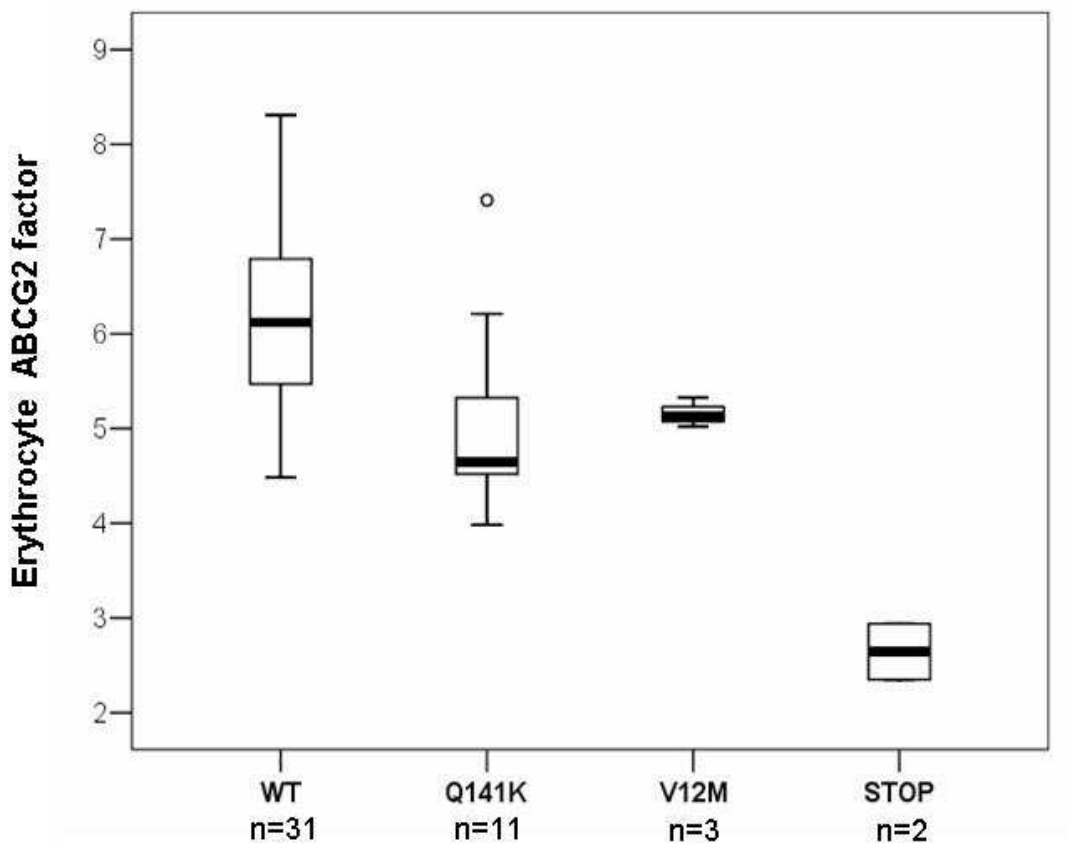

Figure 16. ABCG2 is differentially expressed in the red blood cells of individuals carrying homozygous wild type, heterozygous polymorphic or premature stop codon mutant ABCG2 alleles.
Boxplot presentation showing the median and the 25-75th percentiles, whiskers represent 10-90th percentiles. ABCG2 expression is calculated based on the combined reactivity of anti-ABCG2 mAbs (RBC-ABCG2 factor). Labels: individuals carrying wild-type ABCG2 (WT), polymorphic (Q141K, V12M) ABCG2 alleles, or a heterozygous nonsense mutation (STOP), described in the next chapter.

4.3.3 ABCG2 expression in healthy carriers of *ABCG2* nonsense mutations

Two unrelated individuals showed substantially lower than the average (about 50%) erythrocyte ABCG2 expression (2.65±0.29) (Fig. 16). Sequencing of the entire coding region of the *ABCG2* gene revealed heterozygous mutations resulting in premature termination. A nonsense mutation, causing an arginine to stop codon change at codon 236 in exon 7

(c.706C>T, p.R236X, rs140207606) was found in heterozygous form in proband 1. A small deletion (c.791_792delTT, L264HfsX14) causing frameshift and the truncation of the protein were found in proband 2. Both mutations were described previously (Saison; 2012, Zelinski; 2012).

In order to clarify if a direct relationship exists between the heterozygous nonsense mutations and the erythrocyte ABCG2 expression levels, blood samples from the family members of the two probands carrying these premature termination mutations was obtained. A co-segregation of the reduced erythrocyte ABCG2 expression levels (about 50% reduction) and the respective mutations in the two families were observed (Fig. 17).

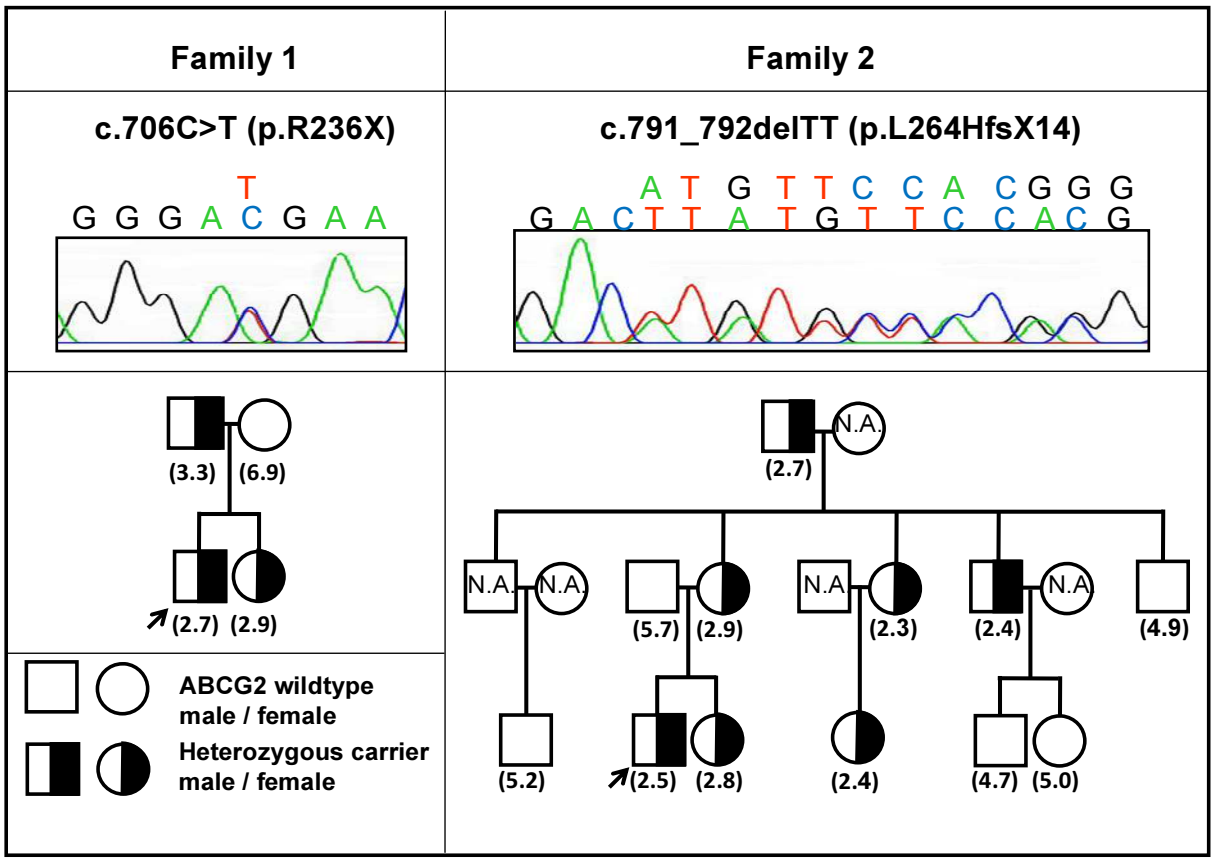

Figure 17. Pedigrees of two families carrying different ABCG2 premature stop mutations – co- segregation of the heterozygous mutation with reduced erythrocytic ABCG2 expression levels. Blood samples obtained from the 14 family members of the two healthy volunteer probands, carrying the premature stop mutations were analyzed for ABCG2 expression and the respective mutations. The RBC-G2 factor values, reflecting ABCG2 expression in erythrocytes, are shown in parentheses. Family members not available for blood donation are labeled by N.A.

4.4 ABCG2 V12M and Q141K polymorphic variants in AML

4.4.1 The effect of *ABCG2* SNPs on AML predisposition

In our study *ABCG2* genotyping was performed on 389 AML patients. Genotype distributions and allele frequencies (AF±95%CI) of *ABCG2* genetic variants in the control and patient groups are presented in Table 15. Distribution of genotypes for the investigated SNPs were in Hardy-Weinberg equilibrium (HWE) within the entire AML cohort and controls (p> 0.05). Minor allele frequencies (AF±95%CI) were not different in AML patients as a whole cohort compared to controls (12M: 4.2±1.4% in AML vs. 4.0±1.9% in controls; 141K: 8.1±2.0% in AML vs. 10.1±3.0% in controls).

Table 15. Genotyping for *ABCG2* genetic variants in the control and patient groups.

Control/patient group	**n**	**AF ± 95%CI** (%)	**Number of genotypes**	**HWE**	**p***
***ABCG2* V12M**					
Control	202	4.0±1.9	186/16/0	0.55	-
AML	389	4.2±1.4	358/29/2	0.10	1.00
AML with normal karyotype	153	5.6±2.6	136/17/0	0.46	0.36
AML with aberrant cytogenetics	218	3.7±1.8	204/12/2	**0.001**	0.58
***ABCG2* Q141K**					
Control	202	10.1±3.0	161/41/0	0.23	-
AML	389	8.1±2.0	330/55/4	0.32	0.13
AML with normal karyotype	153	7.2±3.0	131/22/0	0.34	0.16
AML with aberrant cytogenetics	218	9±2.7	183/31/4	0.06	0.31

Significant p values are shown in bold.
Abbreviations: AF: allele frequency, AML: acute myeloid leukemia; HWE: Hardy Weinberg equilibrium; 95%CI: 95% confidence interval.

Estimated haplotype frequencies (EHF) calculated by the web-based program SNPStats (Sole; 2006) are presented in (Table 16). EHFs of the *ABCG2* gene were not different between cases and controls. *ABCG2* AF and EHF were not different among younger and older AML patients, among patients with different gender, or among AML patients with different etiology (de novo, myelodysplasia or therapy-related AML) (data not shown).

Table 16. Estimated haplotype frequencies of *ABCG2* V12M/ Q141K genetic variants in the control and in the patient groups.

Control/patient group	***ABCG2*** V12M/Q141K		
	12V/141Q	12V/141K	12M/141Q
Control	85.9	10.2	3.9
AML	87.7	8.1	4.2
AML with normal karyotype	87.0	7.1	5.8
AML with aberrant cytogenetics	87.5	9.0	3.5
AML with t(8;21)	69.4	13.9	16.7
AML with intermediate risk aberrant cytogenetics	94.4	4.8	0.8
AML with adverse risk cytogenetics	87.2	10.6	2.2

Abbreviations: AML: acute myeloid leukemia

As AML has heterogeneous acquired genetic background, the following subgroups were analyzed separately: AML with normal (NK-AML, n=153) or aberrant karyotype (non-NK-AML, n=218), MDS-type cytogenetics (n=87), favorable risk cytogenetics (n=66), intermediate risk cytogenetics (n=215), adverse risk cytogenetics (n=90), different recurrent translocations as t(15;17) (n=34), t(8;21) (n=19) or inv(16) (n=13), intermediate risk aberrant cytogenetics (n=62), complex karyotypic changes (n=52), *FLT3*-ITD positive case (n=79), cases with *NPM1* (n=95) or *IDH* mutations (n=59). Further AML subgroup analysis with respect to *ABCG2* SNPs also revealed that *RUNX1-RUNX1T1* [t(8;21)(q22;q22) translocation] positive patients (n=19) showed higher V12M AF (18.4±12.6%) compared to control individuals (p=0.002, allelic model). V12M carriers (12VM and 12MM genotypes) were more frequent (31.6%) in AML with t(8;21) compared to 7.9% in controls (p=0.006; OR [95%CI]: 5.37 [1.80-16.02], dominant model). In AML with intermediate risk aberrant cytogenetics, both *ABCG2* 12V/141K and 12M/141Q EHFs were reduced, (V12M or Q141K carrier frequency was 11.3% in this AML subgroup vs. 28.2% in controls, p=0.006, OR [95%CI]: 0.32 [0.14-0.75]). *ABCG2* Q141K homozygous carriership (141KK genotype) was frequent among AML cases with adverse cytogenetics compared to controls (p=0.01). *ABCG2* AF and EHF were not different in other subgroup set up by the presence or absence of other karyotypic and molecular genetic changes.

4.4.2 The impact of *ABCG2* transporter SNPs on treatment outcome

Treatment outcome was investigated in 307 patients younger than 65 years (167 females/140 males, median age: 47.0, range: 16-62 years,) and treated with curative intention. OS and DFS were not different according to genotype of *ABCG2* in the entire AML cohort, however a tendency was observed indicating a potential survival benefit among patients carrying the *ABCG2* Q141K (141QK and 141KK genotypes). Clinical characteristics and treatment modalities were not different comparing 141K carriers to non-carriers. To further explore the factors potentially influencing the observed tendency toward a survival benefit, subgroup analyzes were performed according to homogenous treatment modalities applied. We analyzed the largest homogenously treated subgroup of AML patients receiving daunorubicin plus cytarabine induction chemotherapy (DNR/AraC, standard '3+7', n=212). *ABCG2* 141K carriers (141QK and 141KK genotypes, n=31) displayed longer OS (48-month OS: 46.7±9.3% vs. 26.9±3.4%; p=0.027) and DFS (48-month DFS: 41.1±9.7% vs. 25.0±3.3%; p=0.019) compared to individuals carrying the major allele (141QQ genotype, n=180).

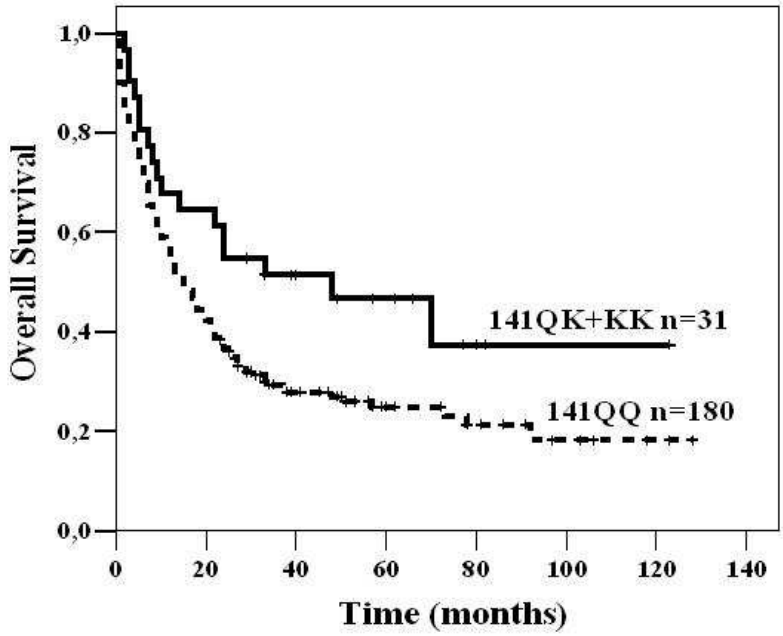

Figure 18. Kaplan-Meier overall survival analysis of AML patients according toABCG2 Q141K mutation. 141QK+KK vs. 141QQ p=0.028.

5. DISCUSSION

AML has a highly heterogeneous acquired molecular background (Dohner; 2010). Cytogenetic alterations detected in the leukemic clone are one of the most important prognostic factors. According to the karyotype, three prognostic categories are distinguished: favorable, intermediate and adverse. AML patients with favorable cytogenetics [i.e., those with t(15;17), t(8;21), or inv(16)] have relatively good response to chemotherapy-based consolidation, whereas patients with unfavorable cytogenetics [i.e. complex alterations] require HSCT to improve prognosis. On the other hand, nearly 50% of AML cases have intermediate risk cytogenetics with normal karyotype (NK) (Gilliland; 2004, Grimwade; 2010). Some NK-AML patients respond well to chemotherapy, but others display adverse outcome. Besides cytogenetics, various acquired molecular genetic abnormalities influence the outcome of the disease (Stone; 2009) and newly identified mutations may provide a deeper insight into the pathogenesis of AML (Figueroa; 2010a). Mutational profiling also helps to improve risk stratification and to bring better founded therapeutic decisions (Patel; 2012).

Twelve years ago, the ″two-hit model″ was proposed showing that there are two main types of mutations occurring simultaneously in AML. Class I mutations cause the inhibition of apoptosis and the induction of proliferation by affecting signal transduction. Class II mutations affect myeloid differentiation (Gilliland and Griffin; 2002). Recent studies revealed mutations that cannot be relegated to any of those two classes, like aberrations affecting epigenetic regulator genes (*IDH1/2, TET2 or DNA-methyltransferase 3A, DNMT3A*) (Thiede; 2012). The program of The Cancer Genome Atlas (TCGA) Research Network has aimed to characterize 10.000 cancer genomes, including 200 AML cases (Ley; 2013). In AML, 260 genes had somatic mutations in at least 2 of the 200 samples; and 154 genes had more than one non-synonymous mutation. Eight different categories of genes (transcription factors, tumor suppressors, chromatin modifiers genes affecting DNA methylation, activated signaling, cohesin complex, spliceosome) were recurrently mutated.

FLT3-ITD affects a receptor tyrosine kinase resulting in activated signaling. It is a very common mutation, mostly occurring in NK-AML patient. The presence of *FLT3*-ITD proved to be an adverse prognostic factor in each cytogenetic risk group. The prognostic impact of *FLT3*-ITD and *NPM1* combination, the *FLT3*-ITD mutational load and the size of *FLT3*-ITD were less intensively studied. The recently identified *IDH* mutations result in altered DNA

methylation through the neomorphic activity of the mutant enzymes. Similarly to *FLT3-ITD*, *IDH* mutations are more common in NK and intermediate cytogenetic risk groups, but their prognostic relevance was less explored.

The treatment outcome depends not only on the acquired mutations in the malignant clone, but also on the inherited genetic variations of the individual patient. Common ABCG2 polymorphic variants may affect drug transport and pharmacokinetics, drug. Therefore, the first part of this study was focused on clinical observation of AML patients regarding to *FLT3*-ITD and *IDH1/2* mutations and the second part on impact of *ABCG2* polymorphic variants or mutations on ABCG2 red blood cell expression in healthy individuals and on treatment outcome in AML patients.

5.1 Impact of FLT3-ITD mutations on the clinical manifestation and treatment outcome in AML

We observed an overall incidence of *FLT3* tandem mutations of 21.3%, which is consistent with previous reports (Gale; 2008, Kiyoi; 2005, Schnittger; 2012). In our cohort *FLT3*-ITD positive patients share distinct clinical characteristics, like association with age (Blau; 2012, Meshinchi; 2008), *de novo* AML (Blau; 2012, Gale; 2008, Schnittger; 2002), M5 FAB subtype (Schnittger; 2002, Thiede; 2002) or higher WBC count (Blau; 2012, Gale; 2008, Thiede; 2002) at diagnosis similarly to previous publications. With regard to cytogenetics, ITD occurred more commonly in the cytogenetically intermediate risk AML and in AML with normal karyotype (Gale; 2008, Thiede; 2002). We also observed an association between *FLT3*-ITD and *NPM1* (Schnittger; 2011, Thiede; 2006).

Generally *FLT3*-ITD mutation is connected to poor prognosis, high relapse rate and reduced survival in AML patients (Advani; 2005, Gilliland and Griffin; 2002, Kayser; 2009). In our study, *FLT3*-ITD alone had no significantly adverse effect on treatment outcome. Beside our data, there are several studies, which did not confirm the adverse effect of ITD on survival (Kiyoi; 2005). The first reason for the lack of association between *FLT3*-ITD alone with survival in our study might be the combination of *FLT3*-ITD with other cytogenetic or molecular alterations, (e.g. *NPM1*). *NPM1*^{pos} and *FLT3*-ITD^{neg} combined mutational status showed the expected favorable impact on survival in our cohort as well (Patel; 2012, Schlenk; 2008). The second cause might be the different therapeutical regiments, such as more frequent autologous HSCT applied in *FLT3*-ITD positive patients and more intensive chemotherapeutical protocols (e.g. autologous stem cell transplantation) in patients with adverse prognostic factors at diagnosis such as *FLT3*-ITD or high WBC. In order to overcome

the bias introduced by the different therapeutical protocols in ITD^{neg} and ITD^{pos} cohorts (namely more frequent autologous HSCT in ITD^{pos} patients), multivariate analysis on survival (including the following parameters; age, cytogenetic risk, HSCT) was performed. It showed that the *NPM1*^{pos} and *FLT3*-ITD^{neg} combined mutational status *(low risk)* is an favorable prognostic factor independently of age, cytogenetic risk, and HSCT as compared to *FLT3*-ITD^{pos} and *NPM1*^{neg}/*FLT3*-ITD^{neg} AML cases *(high risk)*.

For the calculation of *FLT3*-ITD load, peak height or area under the peak curve can be used for the comparison of wild type and mutant allele load. During capillary electrophoresis, the signal intensity can be expressed by either peak height or peak area under the curve and it can be influenced by many factors like; capillary length, capillary failure during electrophoresis, baseline noise, fluorescent dye dissociation etc. The available data considering this issue are quite controversial. One suggestion was that the peak area is more adequate for molecular analysis, because it takes into account the morphology of the peak (Clayton; 1998), on the other hand there are other studies showing no difference between peak height and area.(Issaq and Young; 1977, Leclair; 2004) In our study the correlation coefficient (R^2) between peak height and area was 0.907, indicating a very good correlation. In this analysis peak height values were used for the calculation of ITD load.

In line with several previous publications (Blau; 2012, Kottaridis; 2001, Whitman; 2001) in our study, patients with high *FLT3*-ITD load (>50%) had worse OS compared to those with load ratio below 50%. Possible due to the low number of cases or the interaction with other factors, our data did not show differences comparing patients with load ratio below 50% and those without *FLT3*-ITD mutation, which was shown by (Gale; 2008, Schnittger; 2012). The adverse effect of high *FLT3*-ITD load on treatment outcome can be explained by the presence of segmental uniparental disomy (UPD) (Fitzgibbon; 2005, Raghavan; 2008) covering the *FLT3*-ITD locus. In case of UPD both *FLT3* alleles carry the mutant form. As a result of this *FLT3*-ITD load is higher than 50% and that can be the background of adverse effect giving an extraordinary high survival advantage of the leukemic cells. In connection with this, patients carrying UPD have a worse prognosis and shorter survival time.

Although general agreement regarding adverse outcome associated with high *FLT3*-ITD load exist in the literature, there is a considerable discrepancy in previous publications about the prognostic impact of the length of insertions (Blau; 2012, Kusec; 2006, Meshinchi; 2008, Ponziani; 2006, Schnittger; 2012, Stirewalt; 2006). In a dichotomous analysis, our data supported the observations of studies, which claimed that the longer insertion represented the worst outcome. Interestingly, studies claiming longer insertions as unfavorable chose lower

cut-offs for dichotomous comparisons: 40 bp (Stirewalt; 2006) or 48 bp (Meshinchi; 2008). Studies with the opposite conclusion, namely that shorter insertion resulted in worse impact on prognosis compared to longer insertion, applied higher cut-offs for dichotomous comparisons: 70 bp (Kusec; 2006), 61 bp (Blau; 2012). In addition, several studies found no association between *FLT3*-ITD insertion size and clinical outcome (Ponziani; 2006, Schnittger; 2012). In this study, we observed an unique pattern that patients with medium sized insertions displayed the worst treatment outcomes compared either to patients with *FLT3*-ITD^{neg} status or to the *FLT3*-ITD^{pos} patients with shorter or longer insertions. This explanation overcomes the two previous contradictory observations made by previous groups. It is in line with many recent studies suggesting that not the size of the insertion, but its integration site (affected regions of *FLT3* JMD or TKD1) influences the treatment outcome in AML (Kayser; 2009, Schnittger; 2012). Mutations occurring in TKD1 β1-sheet seemed to associate with reduced remission rates and inferior survival (Kayser; 2009). and two studies revealed a strong correlation between the integration site and the size of ITD: more C-terminal (3´end) located insertions were significantly longer (Blau; 2012, Kayser; 2009). Short ITDs mainly affecting the JMD may cause less extensive damage to the auto-inhibitory function of the JMD, while longer insertion affect interfering with it tyrosine kinase activity.

In summary, in our AML cohort, we confirmed *FLT3*-ITD associations with distinct clinical characteristics. *FLT3*-ITD indicated adverse prognosis considering its combination with *NPM1* mutation or its mutational burden indicative of the loss of the unmutated allele. The most importantly we revealed that *FLT3*-ITDs of medium size may confer outstandingly worst prognostic impact compared to shorter or longer sizes.

FLT3-ITD AML accounts for a distinct subgroup of AML with well-known clinical features. Allogeneic transplantation still seems to be a good consolidation therapy in younger patients, however it is not suitable for elderly patients (Levis; 2013, Levis). For those patients who cannot undergo allogeneic transplantation *FLT3* inhibitors could significantly improve treatment outcome and survival. . It is important to consider not only the presence of the insertion, but also its load and size in order to apply the optimal risk-adapted therapy. *FLT3* inhibitors have been studied for many years, but still there is no such agent which would be improved for AML therapy. One of the reasons for that can be many off-target effects, which cause high toxicity. A few *FLT3* inhibitors were reported as they had a clinical activity; sunitinib, sorafenib, and ponatinib (O'Farrell; 2003, Shah, Zhang; 2008), but recently studied quizartinib seems to give the best results in term of its efficiency to inhibit *FLT3* and it is also tolerable at doses that completely inhibit *FLT3* in vivo (Levis; 2013). According to these

observations the incorporation of *FLT3* inhibitors into the AML treatment would give a significant improvement in the prognosis for AML patients.

5.2 Impact of IDH1/2 mutations on the clinical manifestation and treatment outcome in AML

In our study, AML patients with the *IDH1* or the *IDH2* mutation shared several common clinical characteristics, like manifestation at older age or higher PLT count (Abbas; 2010, Chou; 2011, Marcucci; 2010, Paschka; 2010, Thol; 2010a) at diagnosis. The *IDH* mutations also occurred significantly more often in the cytogenetically intermediate risk AML, similarly to other reports (Abbas; 2010, Boissel; 2010, Chou; 2010a, Chou; 2011, Green; 2010, Green; 2011, Lin; 2012, Mardis; 2009, Schnittger; 2010). Interestingly, in our patient cohort, *IDH* mutations were not associated with normal karyotype. The *IDH* mutations occurred more frequently in AML with normal karyotype in all reports, except for two studies (Chotirat; 2012, Nomdedeu; 2012). Similar to other groups, we also observed an association between *IDH* and *NPM1* (Abbas; 2010, Boissel; 2010, Chotirat; 2012, Chou; 2010a, Chou; 2011, Green; 2010, Green; 2011, Marcucci; 2010, Mardis; 2009, Paschka; 2010, Schnittger; 2010).

We confirmed that R172K mutation showed lower WBC, higher likelihood for having intermediate risk abnormal karyotype compared to R140Q, as well as the lack of co-occurrence with *NPM1* (Boissel; 2010, Chou; 2011, Green; 2011, Patel; 2012). We observed a novel finding in the clinical characteristics of *IDH1* mutations affecting the same codon, R132H and R132C. There was a tendency that the R132H mutation associated more frequently with de novo AML etiology compared to the R132C mutation (90% vs. 50%, p=0.08). In the *IDH1* R132H positive AML, acute myeloblastic leukemia without maturation (FAB M1) morphology was less frequent (p=0.02); the PLT count at diagnosis was higher (p=0.05) and *NPM1* co-occurred more frequently (p=0.02). In our cohort *IDH1* R132H was an independent adverse prognostic factor affecting early death rate and OS, while R132C did not differ from the IDH^{neg} AML samples.

Since *IDH* mutations are described not only in AML, but also in CNS tumors and in myeloproliferative neoplasm (MPN), we prepared a summary table (Table 17) to compare frequencies among *IDH1* codon R132 mutants in those diseases. In the central nervous system (CNS), the vast majority (80-90%) of *IDH1* mutations are R132H (Reitman and Yan; 2010), while R132C is more frequent in haematopoietic clonal disorders. Although the R132H mutation was described to occur in MPN, (Andrulis; 2010b) the reported relative frequency of R132H is lower in the $IDH1^{pos}$ MPN (10.3% vs. 41.1%, p<0.001) compared to the $IDH1^{pos}$

AML (see Table 17 for the review of the literature). There is no difference in relative frequency of R132C between MPN, MDS and AML. The R132S mutation was reported to occur with a higher frequency in MPN. Differences in the observed frequencies of R132H and R132C in CNS tumors, AML, and MPN suggest possible functional variations among *IDH1* codon R132 mutants. Although the ability to produce 2HG was similar in both of the R132 variants, kinetic analyses has shown that the R132C substitution impairs the oxidative decarboxylation of isocitrate to alpha-KG more severely as compared to R132H (Dang; 2009, Gross; 2010, Jin; 2011).

The prognostic impact of *IDH* mutations in AML patients is still under investigation. Several studies have found no prognostic impact of the *IDH2* mutations, (Marcucci; 2010) (Lin; 2012, Paschka; 2010, Thol; 2010a) while others suggested that R140 confers good and R172 adverse prognosis (Boissel; 2010, Chou; 2011, Green; 2011, Patel; 2012). *IDH1* was generally considered as a weak adverse prognostic factor (Zou; 2010), exerting its adverse effect only in special AML subgroups (like *FLT3* ITD negative, (Green; 2010) *NPM1* negative (Mardis; 2009, Schnittger; 2010) or *NPM1* positive (Boissel; 2010)). No significant difference was reported between R132C and R132H substitutions. Controversial data on differences between particular types of *IDH* mutations may be caused by grouping them together, while each of *IDH1* and *IDH2* mutations may have a different prognostic impact.

In the first part of *ABCG2* gene analysis we discovered that *ABCG2* mRNA expression in AML can be influenced by different acquired mutations such as; *NPM1* or *IDH1* and *IDH2*. Both *NPM1* and *IDH* mutants in AML showed a reduced *ABCG2* mRNA expression. An explanation for this observation can be the fact, that *IDH* mutations were shown to induce DNA and histone hypermethylation (Figueroa; 2010a, Lu; 2012) and the methylation of *ABCG2* promoter may lie behind the lower transcript level of this transporter (To; 2008, To; 2006). Other reports discovered a specific association between *NPM1* and *IDH* mutations by clustering samples according to their methylation profile similarities (Deneberg; 2011, Figueroa; 2010b). In the background of increased toxicity in R132H cases marked by the early death rate, the role of *ABCG2* function could be ruled out, as other IDH^{pos} patients also harbored lower *ABCG2* expression. Although the low *ABCG2* expression may be one of the factors resulting in survival advance of *NPM1* or *NPM1* plus *IDH* positive AML, which was described previously (Patel; 2012).

Our results suggest possible distinct clinical features in the *IDH1* R132C compared to R132H and *IDH2* R140 compared to R172 mutations. Different mutations affecting the same codon of *IDH1* might associate with distinct features and prognostic impact. Similarly to *FLT3*, it is important, not only to detect the presence of *IDH* mutation, but also its detailed examination, like detecting its exact position and change it causes on the protein level, in order to choose an optimal therapy. A new *IDH1* inhibitor which blocks production of 2-HG and stops AML cells survival was recently discovered (Rohle; 2013, Chaturvedi; 2013). These data suggest that *IDH1* mutant can be effectively targeted and it will be a next potential therapeutic goal in AML. The availability of a mutant IDH1 protein inhibitor increases the need for specific molecular genetic investigations as the bases of therapy.

Table 17. Reported relative frequencies of *IDH1* R132H and R132C mutations in MPN, MDS and AML.

Study	Disease	Number of patients	Number of *IDH1* R132 mutants	Number of R132H mutants	R132H/ *IDH1* R132 mutants	Number of R132C mutants	R132C/ *IDH1* R132 mutants
(Andrulis; 2010b)	MPN	160	3	3	NA	NA	NA
(Tefferi; 2010)		1473	18	0	0.0%	7	38.9%
(Green and Beer; 2010)		16	3	0	0.0%	3	100.0%
(Pardanani; 2010)		200	5	0	0.0%	4	80.0%
Summary of MPN			**29**	**3**	**10.3%**	**14**	**48.3%**
(Andrulis; 2010a)	MDS	71	3	3	NA	NA	NA
(Rocquain; 2010)		65	2	0	0.0%	2	100.0%
(Kosmider; 2010)		100	2	0	0.0%	0	0.0%
(Thol; 2010b)		193	7	1	14.3%	6	85.7%
(Lin; 2012)		82	2	1	50.0%	0	0.0%
Summary of MDS			**16**	**5**	**31.3%**	**8**	**50.0%**
(Ho; 2010)	AML	257	0	0	-	0	-
(Kosmider; 2010)		41	2	0	0.0%	2	100.0%
(Zou; 2010)		68	5	0	0.0%	2	40.0%
(Schnittger; 2010)		1414	93	6	6.5%	51	54.8%
(Chou; 2010a)		493	27	7	25.9%	10	37.0%
(Chotirat; 2012)		230	20	8	40.0%	6	30.0%
(Boissel; 2010)		520	50	22	44.0%	21	42.0%
(Paschka; 2010)		805	61	28	45.9%	20	32.8%
(Marcucci; 2010)		358	49	23	46.9%	15	30.6%
(Thol; 2010b)		53	4	2	50.0%	1	25.0%
(Green; 2010)		1333	107	54	50.5%	35	32.7%
(Abbas; 2010)		893	55	31	56.4%	15	27.3%
(Ho; 2010)		274	12	8	66.7%	1	8.3%
(Rocquain; 2010)		64	3	2	66.7%	1	33.3%
(Wagner; 2010)		275	29	20	69.0%	5	17.2%
(Lin; 2012)		198	4	3	75.0%	0	0.0%
Summary of AML			**521**	**214**	**41.1%**	**185**	**35.5%**

*/** Possible overlapping in patients cohorts. NA-not applicable, because only R132H was investigated by mutation specific antibody.

5.3 Impact of ABCG2 SNPs on protein expression

5.3.1 ABCG2 protein expression on red blood cell membranes in healthy individuals

We have developed a flow cytometry assay to quantitate the expression of the human ABCG2 protein in erythrocytes. The *ABCG2* gene as the genetic basis of one of the high frequency red-blood-cell (RBC) blood group antigens, encoding Junior (Jun) blood group was recently described. Blood group antigen variants caused by genetic alterations may trigger severe diseases during pregnancy and blood transfusion, such as hemolytic disease of the fetus and newborn (HDFN) or adverse hemolytic transfusion reactions. Membrane proteins, comprising about 30% of the total number of human proteins, are crucial in many diseases, while currently no simple assays are available for the determination of their tissue levels. Human erythrocytes express about 350 different integral membrane proteins, including transporters, receptors and blood group antigens. Using this technique, we observed that individuals harboring heterozygous form of Q141K variant exhibited significantly lower expression of ABCG2 protein of their RBC comparing to homozygous wild-type individuals. We also observed two unrelated individuals showing much lower than the average (about 50%) erythrocyte ABCG2 expression. Sequencing of the entire coding region of the *ABCG2* gene revealed that these individuals carry heterozygous mutations resulting in premature termination. A nonsense mutation, causing an arginine to stop codon change at codon 236 in exon 7 (R236X) in the first individual and a small deletion (L264HfsX14) causing frameshift and the truncation of the protein in the second individual were identified. Both genetic alterations were described in homozygous or compound heterozygous form in the background of Junior blood group negative individuals (Saison; 2012, Zelinski; 2012). As next we tested the blood samples from the family members and we observed a co-segregation of the reduced erythrocyte ABCG2 expression levels (about 50% reduction) and the respective mutations in the two families.

In summary, ABCG2 expression on RBC membrane in healthy individuals correlates with the presence of Q141K SNP and by different nonsense mutations. Although Q141K was found to lead to the lower protein expression and activity in vitro (Kobayashi; 2005, Morisaki; 2005), a lower expression level of the *ABCG2* Q141K variant has not been still confirmed at physiologically relevant sites, given the difficulties in obtaining and processing human tissues. Finding 50% of protein expression in variants resulting in premature protein

termination indicates a general bi-allelic expression pattern for ABCG2, as has been suggested, based on mRNA data (Cusatis; 2006, Kobayashi; 2005, Sissung; 2010). Our observations suggest that the *ABCG2* genetic variants associated with the absence of high frequency blood group antigens may be more common than previously described.

5.3.2 The possible role of *ABCG2* SNPs on disease susceptibility and treatment outcome in AML

In *ABCG2*, 12M (1.7-10.3%) and 141K (4.0-14.0%) were reported as the most prevalent variants.(Cervenak; 2006, Ieiri; 2012) Allele frequencies described in this study were within the range previously reported for Caucasian populations. The genotype distributions for all investigated SNPs were in accordance with HWE in the entire patient and control cohorts. On the other hand, deviation from HWE was found in several AML subgroups: *ABCG2* 12MM homozygous (p=0.001) and 141KK homozygous (p=0.06) individuals were overrepresented in AML with aberrant cytogenetics, indicating that 12MM and 141KK homozygosity may represent a risk factor for AML with chromosomal abnormalities. However, the relatively low frequency of these SNPs in Caucasian populations makes difficult to prove this hypothesis.

ABC-transporters (including *ABCG2*) are involved in cellular protection mechanisms against exogenous and endogenous carcinogens, therefore functional polymorphisms of these genes were implicated as inherited modifiers of cancer susceptibility. Although ABCG2 is expressed on hematopoietic progenitors (Scharenberg; 2002), predisposition for AML was less intensively studied compared to ALL regarding the effect of *ABCG2* variants. *ABCG2* SNPs were investigated less intensively with respect to AML disease susceptibility, with a single report with 112 AML patients defining no association between *ABCG2* Q141K and AML predisposition (Muller; 2008). As we also did not find any association between *ABCG2* V12M and Q141K in the entire AML cohort, we performed subgroup analyses, and revealed the V12M variant may predispose certain, relative rare form of AML with recurrent t(8;21) translocation (5-10% of AML cases).

The advantageous setting of our study to detect even a modest impact in susceptibility involved the relatively large number of investigated individuals, the consecutive enrolment of patients in a single center avoiding the selection bias caused by the exclusion of high risk patients and the detailed haplotype analysis (Jamroziak and Robak; 2008). As AML is rather heterogeneous disease, the clinico-pathologically well characterized patient cohort enabled us to perform subgroup analyses on selected cohorts.

The reports on *ABCG2* SNPs and treatment outcome in AML patients are conflicting. The four previous reports about the impact of *ABCG2* SNPs on treatment outcome in AML are controversial (See Table 18) with no association between *ABCG2* V12M or Q141K and treatment outcome in a small population of 112 AML patients with mixed ethnicity (Muller; 2008). In a study investigating 261 AML patients (80% Caucasian) treated with standard cytarabine and anthracyclin treatment, *ABCG2* V12M carriers (12VM and 12MM genotypes) were reported to show better survival (adjusted HR: 0.44, 95%CI: 0.25-0.79), and increased toxicity (OR: 8.41; 95%CI: 1.10-64.24) (Hampras; 2010). Interestingly, in our patient cohort the t(8;21) translocation positive subgroup (which is a well-known favorable cytogenetic abnormality in AML) carried more often the *ABCG2* V12M variant. In the same study, patients with homozygous *ABCG2* 141KK genotype (n=3) showed an increased hazard of death compared to CC genotype (univariate HR: 2.557; 95%CI: 0.79-8.42), which did not reached the level of significance in multivariate analysis. Another study reported an opposite finding in Chinese patients (n=141 AML, n=43 ALL), where *ABCG2* V12M carriers showed adverse OS (HR: 6.05, 95%CI: 3.58-10.21 without HSCT or HR: 4.00, 95%CI: 1.30-12.34 with HSCT) (Wang; 2011). In this study, all acute leukemia patients were combined irrespective from diagnosis. The reported V12M allele frequency (50.5%) exceeded previous reports from Asian countries (19.3-28.9%) (Ieri, 2012), and the reported genotypes showed a deviation from HWE with the underrepresentation of heterozygous individuals compared to homozygous 12VV or 12MM genotypes. Population differences between our study and this study (Wang; 2011) can be also responsible for the different findings. A recent Italian study (Tiribelli; 2013) reported that the presence of *ABCG2* 421A polymorphism was associated with adverse prognosis in AML patients treated with idarubicin (n=125). Although anthracyclines are generally presumed to be substrates of *ABCG2*, several studies reported differences between daunorubicin and idarubicin. *ABCG2* overexpression resulted in daunorubicine, but not idarubicine resistance (Abbott; 2002). Similarly other group did not find difference between 141K carriers and non-carriers, analyzing only the genotypic data in combination with *ABCG2* expression (Tiribelli; 2013). It is contradictory that 141K carriers, in whom we found impaired *ABCG2* expression, have similar survival compared to patients with high *ABCG2* expression. Contradictory to previous publications our results strongly suggest that Q141K mutation might influence treatment outcome in AML patients differentially depending on chemotherapy applied.

Table 18. Summary of previously published studies about the impact of *ABCG2* on treatment outcome of AML

Study (number of AML patients)	**Population**	**Investigated SNPs**	**Induction treatment**	**Major findings**
(Muller; 2008) (n=112)	Mixed (Jewish/Arab)	*ABCG2* Q141K	DNR + AraC	No correlation with CR, OS
(Hampras; 2010) (n=261)	Mixed (90% Caucasian, USA)	*ABCG2* V12M, Q141K	Anthracycline (type not stated) + AraC	12VM&12MM is associated with higher OS, increased toxicity
(Wang; 2011) (n=141)	Asian (Chinese)	*ABCG2* V12M, Q141K	DNR/MTX + AraC	12VV is associated with higher DFS, OS
(Tiribelli; 2013) (n=163)	Caucasian (Italian)	*ABCG2* Q141K	FLAI/FLAIE	No correlation with CR, RR, DFS, OS; Correlation with DFS and OS, only when analyzed in composition with *ABCG2* expression

Abbreviations: AraC: cytarabine; CR: complete remission, DFS: disease free survival; DNR: daunorubicin, FLAI/FLAIE: fludarabine, cytarabine and idarubicin, with or without etoposide; MTX: mitoxantrone; OS: overall survival, RR: relapse rate.

Our study proves that not only the presence of a mutation, but also its detailed analysis (load and size of *FLT3*-ITD, *IDH1/2* mutations affecting different codons) may result in different treatment outcome in AML patients. ABCG2 expression on RBC membrane in healthy individuals is influenced by the presence of Q141K SNP and by different nonsense mutations. The *ABCG2* Q141K SNPs may prolong survival in AML patients, due to lower *ABCG2* expression and lower multidrug resistance.

During the last few years the genetic background of AML has been intensively studied. AML is known for its molecular heterogeneity. Each of AML patients harbors an unique set of mutations and molecular features characterizing his or her disease (Smith; 2010). Unfortunately new, therapeutical solutions are still lacking. In connection with this, there is a high need for novel drugs, specific inhibitors and more effective treatment. Patel showed that AML patients harboring different mutations, such as *DNMT3A* or *NPM1* had an improved outcome, depending on the induction therapy (Patel; 2012). This suggests that mutational profiling can be important and useful for risk stratification and therapeutic decisions (Patel; 2012). Many new inhibitors specifically targeting some genetic changes in AML are now being developed (Chaturvedi; 2013, Levis; 2013, Rohle; 2013). Also new AML therapies are being tested, including the combination of all-trans retinoic acid and arsenic-trioxid in acute

promyelocytic leukemia or the combination of intensive chemotherapy with *KIT* inhibitors in core-binding factor AML and *FLT3* inhibitors in AML with *FLT3* mutation (Schlenk; 2013). Personalized cancer therapies for individual patients may help to decide about the next line of therapy and in selection of the most optimal targeted drugs and therefore to induce long-term remissions (Pemovska; 2013). With recent, advanced diagnostic strategies in genetic, epigenetic, and metabolic profiling, application of the personalized strategies will hopefully be available in the near future to improve outcomes for AML patients (Allen and Schinkel; 2002, Guzman and Allan; 2014). Therefore detailed characterization of acquired and inherited genetic background in malignant disorders, including AML is necessary in establishing a targeted and a personalized treatment strategy for improving outcome.

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7. SUMMARY

AML has a highly heterogeneous acquired genetic background. Each AML patient harbors a unique set of mutations and molecular features characterizing the disease. Mutational profiling of acquired and inherited variants can be important and useful for risk stratification guided therapeutical decisions. One of the most frequent mutations affects *FLT3* gene: the internal tandem duplication (ITD) in *FLT3* autoinhibitory juxtamembrane region causing a permanent receptor signal transduction and uncontrolled proliferation of leukemic cells. Another recently identified, acquired aberration affects mutational hotspots of isocitrate dehydrogenase 1 and 2 (*IDH1* codon 132 *and IDH2* codons 140 and 172). The inherited polymorphic variants of ATP-binding cassette multidrug transporter, sub-family G, member 2 (*ABCG2* V12M and Q141K) may influence drug transport during chemotherapy.

In this work, I focused on the impact of acquired mutations (*FLT3*-ITD, *IDH1/2*) and inherited polymorphisms (*ABCG2*) on the clinical manifestation and treatment outcome in AML and on impact of *ABCG2* inherited variations on ABCG2 protein expression in healthy individuals.

FLT3-ITD was detected in 21% of AML cases. Patients with higher ITD load or medium size insertions (between 48 and 60bp) showed worse treatment outcome and survival compared to patients with lower load or insertions with other size. *IDH1* and *IDH2* mutations were mutually exclusive, detected in 8% and 7% of AML cases respectively. *IDH1* R132H negatively influenced survival compared to *IDH1/2* wild type or to R132C.

Healthy individuals carrying the heterozygous *ABCG2* Q141K variant exhibited significantly lower expression of ABCG2 protein in red blood cell membrane, as compared to homozygous wild-type individuals. Sequencing of the *ABCG2* gene of two unrelated individuals showing lower erythrocyte ABCG2 expression revealed heterozygous mutations resulting in premature termination (R236X, L264HfsX14). *ABCG2* Q141K positively influenced survival in patients with daunorubicin-based treatment regimens.

Detailed characterization of acquired and inherited genetic variants in malignant disorders, such as AML helps to predict prognosis and to apply risk adapted treatment strategies aiming at improving outcome.

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9. SUPPLEMENTARY DATA

9.1 Supplementary tables

Table S1. *FLT3* and *NPM1* fragment analysis primers. *FLT3* primers were adopted from (Kottaridis; 2001), *NPM1* from (Thiede; 2006).

Name of primer	Sequence of primer
FLT3-ITD-F	5'-GCAATTTAGGTATGAAAGCCAGC-3'
FLT3-ITD-R	5'-5D3-CTTTCAGCATTTTGACGGCAACC-3'
*NPM*1-E12F	5'-GTGGTAGAATGAAAAATAGAT-3'
*NPM*1-E12R	5'-5D2-CTTGGCAATAGAACCTGGAC-3'

Table S2. *IDH1* and *IDH2* HRM primers. *IDH1* primers, *IDH2*-HRM140Q-R and *IDH2*-HRM172K-R were adopted from (Tefferi; 2010).

Name of primer	Sequence of primer
IDH1-HRM-F	5`-CGGTCTTCAGAGAAGCCATT-3´
IDH1-HRM-R	5´-CACATTATTGCCAACATGAC-3´
IDH2-HRM140Q-F	5´-TGGGACCACTATTATCTCTGTCCTC-3´
IDH2-HRM140Q-R	5´-TGATGGGCTCCCGGAAGA-3´
IDH2-HRM172K-F	5´-AAACATCCCACGCCTAGTCC-3´
IDH2-HRM172K-R	5´-CCCAGGTCAGTGGATCCC-3´

Table S3. *IDH1* and *IDH2* control and allele specific primers. *IDH1* primers were adopted from (Chou; 2010a). Mismatches differentiating between the mutation and wild type sequences are marked with bold face characters, while additional mismatches introduced to the further prevention of the amplification of the wild type allele are underlined.

Name of primer	Sequence of primer
IDH1-Fcont	5′-AATGTGTTGAGATGGACGCCTATTTGT-3′
IDH1-Rcont	5′-TGAGAAGAGGGTTGAGGAGTTCAAGT-3′
IDH1-132C	5′-TGGATGGGTAAAACCTATCATCATAG*A*T**T**-3′
IDH1-132H	5′-GGATGGGTAAAACCTATCATCATAG*A*C***A***-3′
IDH1-132G	5′-TGGATGGGTAAAACCTATCATCATAG*A*T***G***-3′
IDH1-132L	5′-GGATGGGTAAAACCTATCATCATAG*A*C***T***-3′
IDH1-132S	5′- TGGATGGGTAAAACCTATCATCATAG*A*T***A***-3′
IDH2-Fcont	5′-TTGGGGTTCAAATTCTGGTTGA-3′
IDH2-Rcont	5′-CCACTCCTTGACACCACTGC-3′
IDH2-140Q	5′-TGTGGAAAAGTCCCAATGGAACTATAC***A***-3′
IDH2-172K	5′-CCAAGCCCATCACCATTGGAA***A***-3′

Table S4. *ABL1* QPCR primers. *ABL1* primers and TaqMan probe were adopted from (Beillard; 2003).

Name of primer	Sequence of primer
Taq-*ABL*-R	5’-GATGTAGTTGCTTGGGACCCA
Taq-*ABL*-F	5’-TGGAGATAACACTCTAAGCATAACTAAAGG
Taqman-*ABL*	5’-/56-FAM/CCATTTTTGGTTTGGGCTTCACACCATT/36-TAMNph/

Table S5. *ABCG2* primers used for genotyping. The position of the polymorphic variants on the sensor probes are marked with bold face characters.

Name of primer	Sequence of primer
V12M-LCF	5’-ATGTATTGTCACCTAGTGTTTGC-3’
V12M-LCR	5’-AGCTCCTTCAGTAAATGCC-3’
V12M-ANC	5’-GCGGGGAAGCCATTGGTGT-/36-FAM/-3’
V12M-SENS	5’-/5Bo650-XN/-CCTTGTGACA**C**TGGGAT-/3Phos/-3’
Q141K-LCF	5’-TATGTATACTAAACAGTCATGGTCTTAGA-3’
Q141K -LCR	5’-TGGAGTCTGCCACTTTATCCAGACCT-3’
Q141K-ANC	5’-GATGATGTTGTGATGGGCACTCTGACGGTGAGA-/36-FAM/-3’
Q141K-SENS	5’-/5Bo650-XN/.-AAACTTA**C**AGTTCTCAGCAGCTCTTCGG /3Phos/-3’

Table S6. *ABCG2* primers used for sequencing. All primers were adopted from (Zelinski; 2012).

Name of primer	Sequence of primer
ABCG2-2F	5'-TCATTGGAAATGAAGCTGCTC-3’
ABCG2-2R	5'-AACAAATGAAAGCATGTGTCTG-3’
ABCG2-3F	5'-TTTAAGAGTTGGTTTGTGCTT G-3’
ABCG2-3R	5'-CTGACATGCGTTGCAAATG-3’
ABCG2-4F	5'-TTGGATTCAAAGTAGCCATGAG-3’
ABCG2-4R	5'-GACCATGTCACATAATCAACTGG-3’
ABCG2-5F	5'-GAACTGCAGGTTCATCATTAGC-3’
ABCG2-5R	5'-TCTCATTGTTATGGAAAGCAACC-3’
ABCG2-6F	5'-CAAGGTATCCACTTATTTGCTG-3’
ABCG2-6R	5'-TTTCACTCCAACAGAAGAGGAAG-3’
ABCG2-7F	5'-TCAGGCTGAACTAGAGCAAAC-3’
ABCG2-7R	5'-TCTACCCAAAGACCAAACAGC-3’
ABCG2-8F	5'-TGTCTTCTCTAGCCTTACCTCCC-3’
ABCG2-8R	5'-CAACAGAAATTCACAAAGCCA C-3’
ABCG2-9F	5'-GGTGTTAGGGAAGCATCCAAG-3’
ABCG2-9R	5'-CCAATAATTGGAAGGGTGGG-3’
ABCG2-10F	5'-GTTGGCCAAGCCATTGAG-3’
ABCG2-10R	5'-TGACTCATCCTACCCTCAATAAAAG-3’
ABCG2-11F	5'-TTTTCCTGCTTAACTTTATACACTGTC-3’
ABCG2-11R	5'-GTAATCCTCCGGATCCCATC-3’
ABCG2-12F	5'-CTTGTTCAACCCCTGCTGTC-3’
ABCG2-12R	5'-TCATGGTTTGGTTTATAGTTTTGAG-3’
ABCG2-13F	5'-AGGGTGGTTGGAGAGTGG-3’
ABCG2-13R	5'-AAGCAGAGCCCCATTTACAG-3’
ABCG2-14F	5'-GCACATGCAGAGGAGAAGAG-3’
ABCG2-14R	5'-TTTCACAGCTCATGGTCAGG-3’
ABCG2-15F	5'-CAATAAAGATTAAACTCAGAGGGC-3’
ABCG2-15R	5'-AATTCAGTGCCCCTGGAAG-3’
ABCG2-16F	5'-GGCTTGGTTCAATTTTAGGC-3’
ABCG2-16R	5'-TGTGCAACAGTGTGATGGC-3’

9.2 Publications